



RESISTANCE TO MULTI ORGAN FAILURE AND METABOLIC ALTERATIONS AFTER
GLOBAL ISCHEMIA/REPERFUSION IN THE ARCTIC GROUND SQUIRREL

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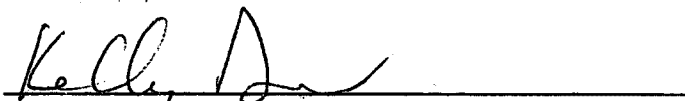
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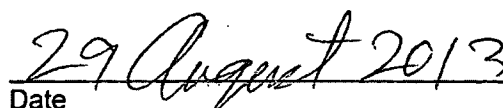

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**RESISTANCE TO MULTI ORGAN FAILURE AND METABOLIC ALTERATIONS AFTER
GLOBAL ISCHEMIA/REPERFUSION IN THE ARCTIC GROUND SQUIRREL**

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

In Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

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Fairbanks, Alaska

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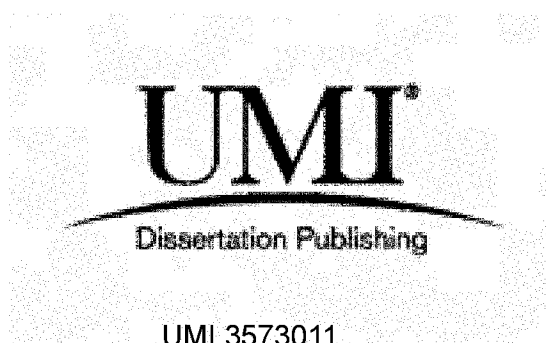
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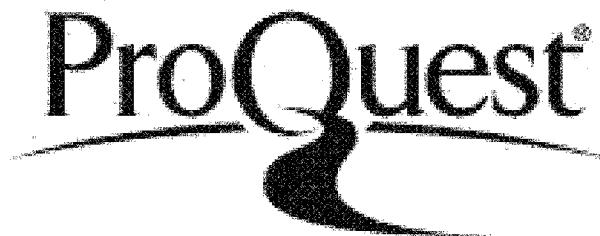


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Abstract

Cardiac arrest (CA) and hemorrhagic shock (HS) are two clinically relevant situations where the body undergoes global ischemia/reperfusion (I/R). Hibernating animals such as ground squirrels have been shown to be resistant to I/R injury in various tissues. The present study compared physiological and metabolic changes occurring during global I/R in an I/R-injury prone animal, the rat, to that of I/R injury resistant animals, arctic ground squirrels (AGS). We sought to determine if AGS are protected from multi organ failure after global I/R and if any protection is dependent upon their hibernation season or the ability to maintain a stable metabolic profile during I/R. For CA, rats and euthermic AGS were asphyxiated for 8 min, inducing CA. For HS, rats, euthermic AGS, and interbout arousal AGS were subject to HS by withdrawing blood to achieve a MAP of 35 mm Hg for 20 min before reperfusion. For both I/R models, the animals' temperature was maintained at 36.5-37.5°C. After reperfusion, animals were monitored for 3 hours (HS) or 7 days (CA), then tissues and blood were collected for histopathology, clinical chemistries, cytokine level analysis (HS only), and ¹H-NMR metabolomics of hydrophobic and hydrophilic metabolites (HS only). For the HS studies, a group of rats and AGS were monitored for three days after HS to assess survival and physiological impairment. Regardless of season AGS showed no physiological deficit 12 hours after HS or CA. Blood chemistries and circulating cytokine levels indicated liver damage and systemic inflammation in the rats while AGS showed no signs of organ damage or inflammation. In addition, rats had a shift in their hydrophilic metabolic fingerprint and alterations in several metabolite concentrations during HS-induced I/R, indicative of metabolic adjustments and organ damage. In contrast, AGS, regardless of season, were able to maintain a ¹H-NMR metabolic profile with few changes in quantified metabolites during I/R. These data demonstrate that AGS are resistant to systemic inflammation and organ damage/failure after I/R and this resistance is not dependent on their ability to become hypothermic during insult but may stem from an intrinsic resistance to disruptions in their metabolic processes during I/R.

Dedication

To my husband and children whose support and encouragement made all the difference.
And to my friends and family, we are all lifelong learners.

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Introduction

Over the course of time, animals have adapted to thrive in their distinct environments. In periods when the environmental factors are not suitable for “normal” life, some animals employ mechanisms that allow them to survive until conditions are again favorable. One such adaptation is hibernation. During times when food is scarce, as occurs in the winter months, ground squirrels enter a state of greatly reduced metabolism and energetic expenditure. The biochemistry of these animals not only enables them to survive through harsh environmental conditions, but also through physiological extremes that would be fatal to other species. In particular, ground squirrels have been found to be resistant to tissue and organ injury after ischemia/reperfusion (I/R). What the specific physiological and/or biochemical mechanisms are allow for the observed resistance to I/R damage have yet to be explained. However, researchers have discovered several possible mechanisms including: a seasonal metabolic switch from carbohydrate to lipid metabolism, protective mechanisms in the small intestine which may inhibit the perturbation of damage to other organs, circulating antioxidants, and/or the ability to become extremely hypothermic. When elucidated, the protective mechanisms utilized by ground squirrels to avoid I/R injury could provide a better understanding of human I/R injury and possible routes to inhibit its detrimental effects.

In humans, cardiac arrest (CA) and hemorrhagic shock (HS) are two clinically relevant situations where the body undergoes global ischemia as blood pressure drops below the threshold at which tissues can be adequately perfused. The studies within this thesis compared the physiological and metabolic changes that occur after global I/R in an I/R-injury prone animal, the rat, to that of I/R injury resistant animals, arctic ground squirrels (AGS). Chapter 1 is a review article detailing the current understanding of systemic I/R injury, the propagation of multi organ failure (MOF), and the ability of obligate hibernators to resist I/R injury at the tissue-specific level. This chapter will be submitted to the journal of Critical Care Medicine.

Chapter 2 compares the physiological and biochemical changes that occur after I/R in a species that is known to be I/R resistant (the AGS) to one that, like humans, are prone to I/R injury (rats). Though comparison, we sought to determine where in the propagation of MOF after systemic I/R, the AGS differed from the I/R injury prone control. These differences may account for how the AGS is able to resist I/R injury. From these studies, we narrowed our comparison to difference in metabolic response during and after I/R. In Chapter 3, ¹H-NMR-based metabolomics was used to further differentiate the responses of the I/R prone rat and the resistant AGS. In this chapter, we used metabolic markers that were present in the rat but not in the AGS as punitive biomarkers of poor prognosis after I/R. Chapters 2 and 3 are also intended for submission to the journal of Critical Care Medicine. As a whole, this thesis examines systemic I/R injury and the propagation of MOF and how obligate hibernators' response to I/R insult may hold the physiological key to establishing a strategy to reduce or eliminate the disastrous effects of systemic I/R insults in I/R injury prone humans.

Chapter 1. Ischemia Reperfusion Injury in Obligate Hibernators¹

From hot to cold and back again, hibernating mammals are capable of acclimating to environmental extremes. Such plasticity provides protection against not only environmental extremes, but also physiological ones. The biochemical mechanisms underlying this flexibility, when fully understood, could provide the foundation for human therapeutics for ischemia/reperfusion (I/R) injury that occurs during cardiac arrest and hemorrhagic shock.

Ischemia/Reperfusion Injury

Several human medical situations can cause I/R injury. Two of the most common are cardiac arrest (CA) and hemorrhagic shock (HS). Both are leading causes of death and disability in the US. CA is especially detrimental. Of the ~700,000 people resuscitated each year following CA, fewer than 40% survive and of those who do, less than 10% return to their previous level of mental and physical function (1). Because of post-CA brain injury, myocardial dysfunction, and systemic I/R injury (2). CA followed by the resumption of spontaneous circulation creates a pathophysiological state of whole-body ischemia when the heart does not beat in a functional capacity that is followed by reperfusion upon successful cardiopulmonary resuscitation.

Cardiac Arrest

Cardiac arrest represents the most severe form of shock in which blood pressure drops quickly before the heart stops and circulations ceases. Systemically, cells quickly become ischemic. Even after ROSC, there is inadequate oxygen delivery to the tissues due to myocardial dysfunction and microcirculatory failure resulting from impaired vasoregulation (2, 3). In addition, there is also impaired oxygen utilization by the tissues and cells (2). The small intestine is particularly sensitive to ischemia, more so than either brain or muscle (4). Although the pathophysiology that occurs after CA I/R is poorly understood, it is dominated by systemic inflammation, including the activation of cytokines (IL-1, IL-6, IL-8, and TNF- α). This is followed

¹ Bogren LK, Drew KL, prepared for submission to Critical Care Medicine, 2013.

by the sequestration of activated neutrophils in the organs and ultimately the development of multi organ failure (MOF) (5).

In contrast to the sudden drop in mean arterial pressure (MAP) that occurs with CA, hemorrhagic shock is characterized by a slow decline in MAP as blood volume decreases (Fig. 1.1).

Hemorrhagic shock

Shock has been called “the rude unhooking of the machinery of life” (6). Worldwide, hemorrhagic shock is the number one cause of death in trauma patients, the majority of whom die from multi organ dysfunction syndrome (7, 8). HS occurs when the blood loss is so great that the decrease in intravascular volume and impaired cardiac preload leads to insufficient perfusion of the organs. This ultimately causes a systemic imbalance between oxygen supply and demand leading to cellular hypoxia (8, 9). After HS, there may be a flow independent impairment of systemic oxygen utilization that occurs from changes in the mitochondria during I/R that disrupts their ability to utilize the available oxygen (10-13). The development of multi organ dysfunction syndrome and MOF has been associated with an inability to utilize O₂ although delivery to the tissue is adequate as is the inability to utilize substrates in oxidative metabolism (14). During the common treatment of HS, which includes administration of 100% O₂ (15), hyperoxia and the generation of free radicals, specifically, reactive oxygen species (ROS), is enhanced (16). ROS that are produced cause oxidative damage to the tissues that ultimately leads to complications such as acute respiratory distress syndrome and MOF (8).

ROS generation during I/R

During periods of ischemia, perfusion of tissues greatly diminishes or ceases. This results not only in a shortage of oxygen, but also a lack of energy substrates and the removal of toxic waste products. If the ischemia persists, ATP levels in a cell can be depleted resulting in the inability to maintain membrane integrity and ion gradients, eventually leading to cell death (5).

However, it is the reintroduction of oxygen during reperfusion and the generation of ROS that ultimately cause the most damage (17).

One method that eukaryotic cells produce ATP is via ATP synthase, the last step in oxidative phosphorylation. The components of the electron transport chain (ETC) are located on the inner mitochondrial membrane. As electrons move from a higher to lower energy states along the ETC, a proton gradient is formed that is ultimately used to drive the synthesis of ATP from ADP and Pi. The last electron acceptor in the chain is oxygen (O_2).

Normally, over 90% of the available cellular oxygen is utilized by the ETC (18). Under ischemic conditions, oxygen is no longer available to act as an electron acceptor. When the ETC chain becomes congested due to loss of the terminal electron acceptor (O_2) and/or decrease in the activity of the complexes within the chain, the half-life of the partially reduced intermediates increases. This increases the likelihood of electrons reacting with O_2 , reducing it to O_2^- (17, 19). Complex I (NADH Dehydrogenase) and complex III are thought to generate most of the O_2^- in the ETC. Approximately 1-4% of the oxygen consumed generates ROS (20). The ROS production is minimized by low intramitochondrial oxygen concentration and the positioning of electron carriers within protein complexes thereby facilitating the flow of electrons, and uncoupling proteins that allow proton leak and prevent a back up of electrons that might reduce oxygen and form ROS.

Although ischemia inhibits the flow of electrons through the ETC, it is reperfusion that is responsible for the disastrous rise in ROS formation. Under normal conditions, the ETC is not 100% efficient and ROS are constantly being generated. During reperfusion there is an influx of oxygen and the unstable ETC complex intermediates lose electrons to this newly available electron acceptor, forming ROS. The ROS generated include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxy radical ($\cdot OH$). The hydroxy radical is particularly cytotoxic but all ROS generated have electrons available that react with cellular components (21). The overall effect of this oxidative stress is damage to the biomolecules within the cell including proteins,

nucleic acids, and lipids and the activation of stress signaling pathways such as JNK and p38 (19, 22, 23). An excess of unmitigated ROS can cause mitochondrial damage including the release of cytochrome c and premature activation of apoptosis (24).

To combat ROS, cells contain a host of antioxidants that are able to neutralize these potentially damaging toxic oxygen metabolites. These antioxidants act as ROS scavengers/quenchers, or by dismutation via superoxide dismutases (25, 26). After ischemia, the breakdown of antioxidants coincides with the neutralization of free radicals (27, 28). Antioxidants have proven protective against I/R –induced organ dysfunction (13, 29-31). However, during I/R injury, the quantity of ROS generated can easily exceed a cell's antioxidant capacity resulting in ROS damage. If the damage is great enough, the cell will die. Widespread cell death can ultimately lead a systemic inflammatory response and MOF.

Multi Organ Failure

MOF is unique in that the organs that fail or become dysfunctional are not those directly injured in the original trauma. In MOF, tissues sustain damage over time by an overwhelming dysfunctional systemic inflammatory process that is still incompletely understood (Fig. 1.2) (32, 33). Generally, the lungs are first to fail followed by the coagulation system, heart, liver, and kidney. Infection is present in only a minority of MOF patients (34). Rather, the elevated levels of cytokines, tumor necrosis factor - alpha (TNF α), Interleukin -1 (IL-1), and Interleukin- 6 (IL-6) in plasma that correlate with a poor prognosis are generated by reperfusion of the ischemic small intestine (8, 32, 35-37)

I/R injury to the small intestine has emerged as a possible crux to development of MOF after HS (34). Initially, the presumed role of the small intestine in inflammation and MOF was that upon I/R injury, the endothelial barrier of the lumen of the small intestine was compromised, allowing the gut contents, including bacteria, to enter into the blood stream. It was these foreign bodies that were then presumed to initiate the inflammatory process (34). However, it has since

been found that when the endothelial cells of the small intestine are damaged by I/R, there is a change in the contents of the mesenteric lymph. These changes result in the formation of danger associated molecular patterns (DAMPs) and cause the contents to act as proinflammatory mediators. After I/R, these mediators are transported through the lymphatic system where the contents of the mesenteric lymph then enter the circulation near the lungs through the thoracic duct (34). Once in circulation, the contents initiate a massive, unregulated inflammatory response that damages the other organs, causing them to fail.

Experiments have been conducted where the mesenteric lymph duct is ligated to prevent its contents from entering circulation prior to hemorrhage and reperfusion. After I/R injury, the contents of the mesenteric lymph are collected. When the lymph duct is ligated, the animal does not develop an inflammatory response nor MOF after I/R. However, when the lymph contents from an I/R-treated animal are injected into the circulation of a naive animal, that animal displays an inflammatory response and subsequently develops MOF (34, 38). Current research is focused on identifying what components of the I/R mesenteric lymph contents are responsible for the inflammatory response.

Thus far, investigators have identified both lipid and protein components of the mesenteric lymph after I/R that spark the inflammatory process. Phospholipase A2-derived neutral lipids are responsible for the priming of neutrophils (39) while glycosylated albumin is the key cytotoxic protein (40, 41). There is also a lipid factor distinct from glycosylated albumin that is cytotoxic. Its identity is currently under investigation (34, 42). It has also been established that pancreatic proteases are required for the formation of mesenteric lymph contents after HS to induce an immune-inflammatory response (38, 43). It has been proposed that the modification of host proteins, such as albumin, via pathways set in motion during I/R induce an immune-inflammatory response in a manner similar to bacteria by acting as danger-associated molecular patterns and binding to “danger signal” receptors on immune cells and activating them (44).

On a larger scale, the mucus layer that covers the intestinal mucosa may also play a role in the development of MOF. Trauma-induced HS causes changes to the intestinal mucus layer. These changes include a decrease in mucus thickness, decreasing the luminal surface area covered by the mucus layer, and a loosening of the mucus structure. Such modifications are associated with an increase in gut permeability, injury to the villi, and enterocyte apoptosis. The alterations in the entire mucosal layer are maximal at the end of reperfusion. In contrast, the changes in the mucus layer are present at the end of reperfusion but become more severe as time after reperfusion progresses (45).

The mucus layer is made up of mucins. The mucins are thought to function as both a physical and chemical barrier to potentially harmful substances contained within the gut lumen from coming into contact and damaging the mucosal epithelium of the small intestine (46). High levels of IgA in the mucus bind bacteria thus modulating bacterial adherence and subsequent translocation (47). In addition, mucins act as antioxidants and free radical scavengers (48, 49) and could provide protection against ROS that are generated during I/R.

How HS disrupts the mucus layer is not known. However, it has been shown that as mucins scavenge oxidants, they lose their viscoelasticity (49). In addition, pancreatic proteases are better able to cleave mucins when the mucins have been modified as may occur during I/R (50, 51). Pancreatic proteases are necessary in creating a MOF-inducing mesenteric lymph content after HS (38). Pancreatic duct ligation prior to HS has been found to alter mucus layer coverage and thickness (38). It could be that these proteases are required to break down the mucus layer, allowing lumen contents to contact the I/R damaged epithelial layer and inducing further damage and ultimately creating a bioactive mesenteric lymph content. When mucus restoration after I/R was inhibited, the effect of proteases on gut injury and permeability was exacerbated (52). In addition, exfoliated epithelial cells in the mucosal layer may aid in mucin degradation as these cells contain glycosidases that are able to remove sugars from mucin (50).

Estrogen levels in rats have also been shown to correlate with preservation of the intestinal mucus layer during HS and reduced intestinal injury from I/R (53). The minimal level of protection was found to coincide with the diestrus phase where estrogen levels are lowest (54). In addition, ovariectomy abrogated protection against HS-induced injury in the small intestine (55). However, these studies did not address if it was the effects of estrogen or the increase in mucus layer thickness that were protective. Estrogen itself plays a protective role in protecting the gut from HS I/R injury via one or both estrogen receptors, ER α and ER β (56).

Ultimately, the current model for the development of MOF after I/R is that reperfusion and reoxygenation of the ischemic gut after HS-ischemia induces the release of signaling molecules into circulation through the mesenteric lymph. These signals act to promote an unregulated systemic inflammatory process that leads to distant organ injury and failure.

Ground Squirrel Protection against I/R Injury

During inhospitable times, many animals have adapted to enter a physiological state where “normal” life is halted and energy consuming activities and processes are suppressed until conditions become favorable. Although there are numerous variations employed by different species including estivation, freeze tolerance, and (20), hibernation is the tactic most widely used by mammals. During hibernation, energy expenditure can be greatly reduced compared to euthermia (57) by diminishing movement, lowering heart rate and breathing to only a few per minute (58) decreasing organ perfusion rates to <10% of euthermic levels (59), and maintaining an isoelectric EEG (60). One of the most extreme examples of this occurs during the torpor bouts of the arctic ground squirrel (AGS; *Urocitellus parryi*).

This small mammal, weighing ~700g, is able to survive winter burrow temperatures reaching -23°C (61) and body temperatures (T_b) dipping below freezing (-3°C) (62). AGS have a circannual rhythm distinguishing between the summer/active and winter/hibernation seasons. Only during the winter can AGS enter a torpid state (Fig. 1.3) (63). Recently it has been shown

that activation of the adenosine A1 receptor in the central nervous system is necessary and sufficient for AGS to enter torpor (64). Torpor bouts last for 1-3 weeks (65) and are interrupted by interbout arousals (IBA) lasting ~10-15 hrs (66, 67) where body temperature returns to euthermic ranges. During torpor bouts, the AGS reduces its metabolic demand to a fraction of basal metabolic rate (68, 69) leading to an overall winter energetic savings despite the drastic energy expenditure incurred for each arousal and euthermic IBA (70).

During torpor bouts, metabolism is depressed (O_2 consumption) which is followed by a gradual drop in body temperature (T_b) (71, 72). The decrease in metabolism to as low as $0.1 \text{ mL } O_2 \text{ g}^{-1} \text{ h}^{-1}$ corresponding to 1-2% of basal metabolic rate (61, 68, 69) allows AGS to survive during the long, harsh arctic winter on fat stores acquired during the late summer months. Although the reason for the energetically-demanding rewarming process of arousals that require 300-fold increase in oxidative metabolism (73) remains elusive, one hypothesis is that during torpor a metabolic imbalance occurs and biochemical reactions required to correct this imbalance can only occur at normal body temperatures obtained during each IBA (65, 74, 75). Studies have found evidence to support this hypothesis (63, 76). However, the experiments to define a distinct cause-and-effect relationship have not been completed, most likely due to the complexity of the issue and the biological system involved. However, studies have established a clear metabolic switch from summer/euthermic carbohydrate metabolism to winter/heterothermic lipid metabolism (63, 77-79).

During the winter hibernation season, AGS do not eat and derive their energy primarily from white adipose tissue as shown by respiratory quotients (RQ) of ~0.7 at ambient temperature between -10 to +8 °C during steady torpor (61, 80). This requires a switch from carbohydrate to lipid metabolism (81) by inhibiting glycolysis and increasing beta oxidation (82). This metabolic fuel switch is the result of a circannual rhythm that is currently described by a “two switch” model (Fig.1.3) (63, 76). The first switch is the metabolic and physiological alterations that occur during

the summer /winter transitions thought to be regulated by gene control. The second switch occurs between torpor and intermittent arousals and relies on protein modifications(78).

Euthermic, AGS have been shown to maintain chronic low-level hypoxia (83). In contrast, during torpor, although the heart rate and breathing are dramatically depressed, the corresponding decrease in metabolic rate allows tissues to be adequately perfused and oxygenated. In hibernating animals, arterial oxygen tension (P_{aO_2}) and tissue lactate measurements are sometimes greater than those of euthermic animals (74, 83-86). During this time, there is no increase in iNOS, a marker for cellular stress. And in the brain, a tissue especially susceptible to ischemic damage, there is no evidence of oxidative damage or histopathology (83). However, during the rewarming stage of arousal, metabolism and oxygen consumption increase at a rate that can greatly exceed that of both torpid and euthermic animals (87). During this time, P_{aO_2} and hemoglobin oxygen saturation (SO_2) are reduced (83) resulting in periods where tissues may become ischemic and then are subsequently reperfused as heart rate and ventilation increase.

During arousals, the brain, heart, lungs, and brown adipose tissue are the first tissues to reach euthermic perfusion levels (when heart rate reaches between 100-200 bpm). The more peripheral and posterior regions, including the splanchnic organs such as the small intestine, are reperfused secondarily (after heart reaches 200 bpm) (58, 88, 89). Despite these episodes of heterothermic rewarming and reperfusion that occur with IBA, AGS do not incur tissue damage (83). Throughout rewarming at an ambient temperature of 2°C, RQ values of AGS were ~0.7, indicating lipids as a fuel source (90). In addition, ascorbate levels were negatively correlated with peak oxygen consumption during arousal. Both byproducts of fat metabolism (ketone bodies) and ascorbate (an antioxidant) are known to combat cellular stress (91-97). However, mechanism(s) underlying the innate protection of AGS against tissue damage during arousal are still being investigated. Possible mechanisms for protection in these animals include 1) a seasonal metabolic switch from carbohydrate to lipid metabolism that would decrease serum

lactate levels while increasing circulating ketone bodies and free fatty acids, 2) protective mechanisms in the small intestine which may inhibit the perturbation of MOF, 3) circulating antioxidants, 4) hypothermia, or a combination of two or more of these mechanisms. When elucidated, these protective systems could provide valuable new therapeutics for human I/R injury.

Metabolic Switch

Obligatory hibernators such as the ground squirrels have been shown to resist I/R injury in liver, heart, brain, and small intestine during the hibernation season when there is a switch from carbohydrate metabolism to lipid metabolism for cellular energy supply (78, 79, 98-104). This metabolic switch limits anaerobic metabolism and the formation of lactate, a herald of poor prognosis and MOF after I/R injury (8, 105). In addition, the increase in circulating lipids and lipid metabolism generates ketone bodies and activates peroxisomal proliferating-activated receptors (PPARs), both of which have been shown to protect against I/R injury.

Lactate

Lactate has long been an indicator of poor prognosis after I/R injury (8, 105). Both blood lactate levels and base deficit are good predictors of mortality, even when other vital signs have been stabilized (106). If serum lactate levels are normalized (≤ 2 mmol/L) within 24 hours after trauma, survival is 100% (107). However, survival decreased the longer it took to establish normalization and after 48 hrs of unresolved hyperlactemia, only 14% survived (105).

Ultimately, the increase in circulating lactate levels, hyperlactemia, stems from both an increase in lactate production and a decrease in removal by the kidneys (108). Lactate is formed under anaerobic conditions when pyruvate, instead of being converted into acetyl-CoA and entering the tricarboxylic acid (TCA) cycle, is converted into lactate to generate NAD⁺ needed for glycolysis to continue. Interestingly, in the case of I/R injury from cardiac arrest, increased serum lactate levels have been proposed to be due to administration of epinephrine during CPR and

epinephrine-stimulated Na⁺, K-ATPase activity in skeletal muscles and not from hypoperfusion-induced anaerobic glycolysis (4, 109, 110). In addition to being a marker for rates of anaerobic metabolism, lactate also generates ROS (111). In vivo, lactate increases ROS production and up-regulate over 600 genes, many of which are regulated by ROS and Ca²⁺ and induce mitochondrial biogenesis (112).

Multi organ failure is caused by a dysfunctional systemic inflammatory process initiated by the small intestine (8, 32). Micordialysis studies have found that there is an increase in lactate in the gut lumen during ischemia (113-115). Lactate increases interleukin- 1 β (IL-1 β), IL-6, and TNF- α (116, 117). Elevated plasma levels of proinflammatory cytokines TNF- α , IL-1 β , and IL-6 indicate a poor prognosis in animal and patient studies of MOF (8). TNF α and IL-1 β mediate neutrophil infiltration into tissues and organs(118-121). Activated neutrophils damage organs and tissue via release of proteolytic enzymes, ROS, and vasoactive substances (119, 122). These cytokines have been shown to cause lung damage in rats after I/R (hemorrhagic shock) (123).

High concentrations of lactate in the blood may constitute an adaptive protective mechanism during times of physiological stress. Lactate can be utilized by the brain as an energy substrate by converting it to pyruvate which is further oxidized by the TCA cycle and oxidative phosphorylation in the mitochondria (124, 125). Lactate levels in tissues and in plasma were found not to increase during hibernation. Concentrations of lactate in tissues (liver, brain, ventricle, skeletal muscle) do not increase, indicating that these tissues are adequately perfused and oxygenated throughout the hibernation period (126). Also, animals do not become hypoxic during hibernation (74). However, these results may also indicate that any lactate generated is consumed, perhaps by a different tissue, such as the brain. Administering low levels of lactate (4 mmol/L) to hippocampal slices after oxygen-glucose deprivation, an in vitro experimental model of ischemia, neurons are protected. In vivo treatment with lactate (2 μ l of 100 mmol/L) after reperfusion also affords neuronal protection resulting in decreased neuron death and better

neurological outcome (127). Increased lactate levels may provide an energy substrate for the ischemia-sensitive brain during times of low oxygen levels.

Ketone Bodies

During the hibernation season, ground squirrels enter a fasted state in which they rely on lipid metabolism for energy. Lipolysis of adipose reserves leads to the release of free fatty acid (FFA) into the blood stream. The FFAs are then transported to the liver where they undergo beta-oxidation to form acetylcoenzyme A (CoA). Two acetyl-Co A fragments condense when hepatic CoA concentration declines, forming the ketone bodies acetoacetate and D-betahydroxybuterate (DBHB). The ketone bodies are then transported to other tissues where they can be taken up and utilized as fuel. Both serum FFA (128) and ketone bodies (129) increase during hibernation. In liver, concentrations of DBHB were found to be 6.5-fold higher during entrance into torpor and 5.4-fold higher during torpor than during the summer active season (104). During the start of hibernation in thirteen lined ground squirrels, monocarboxylic acid transporter 1 (MCT1), a ketone transporter, is upregulated at the blood brain barrier. Glucose and DBHB were taken up into both the heart and brain during torpor. However, both tissues preferentially utilized DBHB over glucose as an energy source (99).

Even though fasted and hibernating squirrels (*S. beldingi*) are ketotic compared to fed animals, only the torpid animals have higher blood DBHB and total ketone concentrations than aroused or euthermic, fed animals (130). The plasma levels of DBHB (1.45 +/- 0.12 mM), acetoacetate (0.21 +/- 0.03 mM), glucose (8.1 mM), and FFAs (0.30 +/- 0.02 mM) all remain constant with no significant changes during torpor. During arousal, DBHB drops to 0.67 +/- 0.10 mM, acetoacetate remains the same, glucose decreases to 4.5 mM, and FFAs increase to 0.49mM. These levels remain constant for the first 8 hours after arousal (130).

Ketones are known to be protective against I/R injury but the mechanism has not yet been elucidated (131). However, they have been shown to decrease injury to neurons subjected

to ischemia (132, 133) by increasing NADH oxidation in the mitochondria and inhibiting ROS production and oxidative stress (134). Additionally, ketones reduce oxidative stress in cardiac tissue (92, 93), and when administered during resuscitation after hemorrhagic shock, survival time is increased (91). A ketogenic diet prevents cerebral ischemic neurodegeneration after cardiac arrest in rats (135) and increases tolerance to cardiac ischemia and enhances recovery of cardiac function after reperfusion (136). Notably, there is an increase in mitochondria in ketotic rats (136).

When DBHB is the major energy source, as during I/R, generation of excess lactate and H^+ is inhibited (137-139). Catabolism of DBHB yields two molecules of acetyl-CoA that can directly enter the TCA cycle. Unlike glucose catabolism, this process does not generate lactate and avoids lactic acidosis under anaerobic conditions. In addition, DBHB yields ~31% more energy than pyruvate as a result of the redox state of the ETC complexes (92). As a result, more ATP can be generated with less oxygen consumed (93). This would be advantageous under ischemic conditions. When DBHB and melatonin are given together to rats after hemorrhage (60% blood removal) survival was prolonged (91). However, in these experiments, the animals were allowed to cool to an average body temperature of ~28° C. Thus, the effects of DBHB that aid survival, if any, may have been augmented by hypothermia, which itself is protective during ischemia/reperfusion injury (140, 141).

Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are transcription factors activated by FFAs. During the winter season, lipid metabolism generates high levels of FFAs in ground squirrels that may activate PPARs (PPAR α , PPAR β , and PPAR γ) (142). When activated, PPARs regulate the transcription of genes involved in lipid and carbohydrate metabolism, inflammation, and expression of mitochondrial uncoupling protein 1 (UCP-1) (143). There is an increase in fatty acid metabolism and a decrease in glucose metabolism. During starvation, PPAR α depresses

pyruvate dehydrogenase complex (PDC) via upregulation of pyruvate dehydrogenase kinase 4 (PDK4), thus blocking entrance of glycolytic products into the TCA cycle (144, 145).

Activation of PPARs also decreases inflammatory gene expression. Specifically, they suppress nuclear factor kappa-beta (NFK β) activity and target genes of nuclear factor of activated T cells (NFAT), activator protein 1 (AP1), and signal transducers and activators of transcription (STATs) (144, 146). PPARs also block expression of inflammatory cytokines (IL-6) and have been shown to induce apoptosis of macrophages exposed to TNF α and interferon- γ (146, 147).

Additionally, activated PPARs induce the expression of mitochondrial uncoupling protein 1 (UCP-1) (145). Mitochondrial UCP-1 has the potential to reduce ROS produced via the ETC when there is a sudden burst in oxygen levels (as occurs during reperfusion). UCP-1 works by allowing hydrogen ions to reenter the mitochondrial matrix, uncoupling electron transport and oxygen consumption from the production of ATP. This prevents the reduction of ETC-intermediates and increases in ROS generation. However, UCP-1 is only present in BAT, and therefore cannot explain how other tissues may be protected. mRNA for UCP-2 and UCP-3 are higher in white adipose tissue and skeletal muscle, respectively, during hibernation in AGS (148). However, increased levels of UCP-3 protein were found not to affect on proton leak kinetics in AGS skeletal muscle (128). In contrast, UCP-3 reduces ROS production in the skeletal muscle mitochondria of *upc3* knockout mice (149). The role of UCPs in the mitigation of ROS generation in ground squirrels has not yet been examined.

PPARs protect against I/R injury. PPAR γ activation reduces systemic inflammation, lung injury (150, 151) and liver apoptosis after hemorrhagic shock (152). By suppressing inflammatory gene expression, PPAR γ agonist activation inhibits endothelial inflammation and improves endothelial dysfunction (153). PPAR γ agonist treatment has also been shown to decrease mucosal injury during I/R and protect against intestinal injury (154, 155).

PPAR activation may be a factor in the ground squirrel's innate protection against I/R injury. During the winter season, PPAR γ expression is altered in ground squirrel intestine and may contribute to the I/R-resistant phenotype (156). In addition, after cardiac I/R, hibernating ground squirrels had reduced damage to myocardial tissue, which was correlated with elevated PPAR α -induced myocardial fatty acid utilization, reduced NF κ B activity and, reduced levels of circulating inflammatory cytokines (TNF α and IL-6)(104).

Antioxidants

There is evidence of oxidative stress upon the tissues during arousal from torpor. Plasma urate levels (indicative of xanthine dehydrogenase conversion to xanthine oxidase induced by oxidative stress) are lowest during torpor and peak during arousal when oxygen consumption rates are maximal (90). Conjugated dienes are increased in the intestinal mucosa of winter-season ground squirrels and may reflect an increase in ROS and subsequent damage to cellular lipids during arousal (157). Additionally, protein ubiquitination increases during torpor. Protein ubiquitination can be used as a marker of protein damage, perhaps by ROS. However, an increase in ubiquitinated proteins during torpor may not be due to an increase in protein ubiquitination, but rather, a decrease in rates of protein degradation due to low T_b (78). Importantly, AGS are a unique exception to this as no evidence to date indicates that they experience oxidative stress during hibernation or after arousal. This may be due to the limited tissues studied in the AGS, but could also be an adaptation to their more extreme hibernating conditions (158). Overall, although there are markers of oxidative stress during torpor and arousal in most ground squirrel species, the tissues remain undamaged due to a variety of potential protective mechanisms.

The process of aerobic metabolism itself generates ROS at a basal level. To combat these reactive molecules, aerobic organism, including AGS, have developed antioxidant defense systems that are able to neutralize the majority of the ROS produced under normal conditions.

Antioxidant defenses in ground squirrels during hibernation include: ascorbate, an extracellular defense; melatonin; intracellular superoxide dismutases (SODs); and catalase.

Ascorbate is well-known antioxidant that, in mammals, is synthesized in the liver from glucose and transported throughout the body. It is the most important antioxidant in plasma (159) and has been shown to minimize oxidative damage from I/R in *in vivo* and *in vitro* cerebral I/R models (94, 95, 97, 160, 161). During hibernation in AGS and thirteen lined ground squirrels, ascorbate levels increase 3 to 4 fold and return to normal within 12 hours after the start of arousal (162). The maximal rate of decrease in plasma ascorbate levels occurs when oxygen consumption is greatest, during arousal. During this time, liver and spleen ascorbate levels increase whereas brain ascorbate levels remained unchanged. It is hypothesized that ascorbate could provide protection against oxidative damage during arousal when tissues are subject to ischemia/reperfusion stress (90).

Melatonin is another endogenous molecule that may provide antioxidant protection. Although melatonin has only shown modest antioxidant activity *in vitro*, it has been shown to aid in ischemia/ reperfusion (163). Recently, it was shown that that melatonin administered after hemorrhagic shock in rats prolonged survival (91). Although normally produced only in the pineal gland, during arousal from hibernation, there are transient high levels of melatonin produced by multiple tissues (59).

In addition to melatonin and ascorbate, in hibernating hamsters, there is also a 3- to 4-fold increase in plasma superoxide dismutase and catalase activities during arousal that aid in scavenging ROS and reducing oxidative damage during this period (164, 165).

Hypothermia

Hypothermia can also play a significant protective role during I/R. The damage induced from I/R and other insults, such as traumatic brain injury, is reduced when body /brain temperature is lowered. Low T_b during torpor reduces the rate of the reactions that could form

ROS. Indeed, decreases in temperature lowers free radical production and may improve neuronal survival (166-169). During arousal AGS resist damage to hippocampal CA1 and cortical neurons even with a 50% decrease in oxygen-hemoglobin saturation and arterial partial pressures as low as 7 mm Hg (170). However, AGS are also tolerant of global cerebral ischemia during cardiac arrest during euthermia when brain temperatures are maintained at 37°C (171). It is important to note that many of the I/R experiments conducted on hibernators, including ground squirrels, allow the animals to cool during ischemia and/or reperfusion making it difficult to untangle innate protection from that conferred by a lowered body temperature (91).

Depressed Immune Function

Systemic inflammation and circulating leukocytes are intricately associated with the pathogenic mechanisms and progression of MOF following ischemia/reperfusion injury. One hypothesis is that during I/R, normal biomolecules are chemically altered to form danger-associated molecular patterns (172). These altered molecules are then thought to become neoepitopes presented on cell surfaces of ischemic tissues or released into circulation, triggering an immune response (173, 174). The activation of the immune system and the recruitment of immune cells to the tissues causes further damage and propagates the pathology initiated by the I/R event.

During torpor, the immune system is suppressed and the number of leukocytes in circulation drops 100-fold (90, 175). This decrease is constant among cell types (85, 175). The numbers quickly return to euthermic levels upon arousal (90, 175-177). The cause of hibernation-induced leucocytopenia is not known but may be due to a decrease in the production and/or maturation of these cells, and/or an increase in their apoptosis or necrosis, or an increase in plasma cytokines and/or chemokines that influence leukocyte trafficking and lead to the sequestering and retention of these cells into the peripheral tissues during torpor (178). However, it is unlikely that there is a disruption in the turnover rate of these cells because rates of cellular processes are greatly reduced at low body temperature during torpor. In support of the

sequestering hypothesis, lymphocytes have been found in the gut and spleen of torpid animals (179). Additionally, there is an increase in ICAM-1 expression and migration of leukocytes in the vascular epithelium, effectively removing them from circulation before arousal and the accompanying I/R (176). Although it has yet to be clearly demonstrated, most likely the decrease in circulating immune cells during torpor stems from their movement into organs and tissues rather than cell death.

In addition to leucopenia during hibernation, few antibodies are produced in response to antigens. This down regulation of antigenic response occurs independent of the low body temperature of torpor (180, 181). The lack of immune response and the reduction in circulating leukocytes that occurs during torpor may play a role in attenuating injury they may incur during rewarming. However, as the immune system reestablishes itself within hours of arousal (178), it is unlikely that it plays a significant role in the innate protection observed in IBA and summer animals.

Small Intestine Protection during I/R

During arousal, the small intestine is one of the last organs to be reperfused (88). Thus, any circulating antioxidants may be reduced or depleted by the time in the arousal and rewarming occurs in the small intestine. In addition, although cell division and migration in the villi epithelium of the small intestine is suspended during torpor, it resumes during each arousal (182-184). It might seem that such metabolically active cells would be more sensitive to disruptions in oxygen supply. However, the gut mucosa of the small intestine from IBA hibernators was found to incur less damage after I/R than summer animals or rats. Damage in the summer animals after I/R included a loss of villi epithelium and structure, increased number of present immune cells, and a congestion of blood vessels. The IBA hibernator's protection against damage from oxidative stress was found to be independent of depressed body temperature (101).

There are several possibilities for why the IBA hibernator's intestine is protected from I/R injury. One of the key enzymes in ketone body synthesis, 3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2 (HMGCS2), is present in higher levels in the intestine of IBA compared to summer ground squirrels (103). Although ketones are normally produced in the liver, fasting induces HMGCS2 in rodent intestine (185). There may be an increased capacity for ketogenesis in the small intestine of IBA hibernators thus allowing for the possibility of localized and temporally relevant ketone body I/R protective capacities.

Albumin levels increase in the intestine of winter season animals and may be due to reduced food intake as fasting has been shown to increase intestinal albumin in mice (186). Post-translational modifications after I/R are different in summer versus winter animals(103). Considering that modified post I/R albumin was a key mediator in the development of MOF after I/R injury (41), it could be that the difference in summer versus winter albumin modulates the inflammatory response and decreases intestinal injury in the winter animals. However, experiments to determine this have not yet been conducted. Aside from its role as a mediator of MOF after I/R, intestinal albumin has both anti-oxidant and anti-inflammatory properties; acting as a free radical scavenger and reducing lipid peroxidation (187). Albumin may also act as a strong ion, counteracting acidosis that occurs from lactate production (188).

During hibernation, there are greater mucosal IgA levels (189). Miner et al. found that high levels of IgA in the intestinal mucus binds and traps luminal bacteria, effectively protecting the intestinal mucosa from bacterial invasion(47). The higher levels of IgA during hibernation may contribute to the innate protection that ground squirrels have against I/R intestinal damage during this time.

Overall ground squirrels show reduced injury to I/R that may arise from a seasonal switch from carbohydrate to lipid metabolism, resulting in an increase in ketone bodies, a decrease in lactate formation, activation of PPARs, high antioxidant levels, low T_b , decreased immune

function, and/or the protection of the small intestine may also contribute to protecting ground squirrels against I/R injury during torpor (Table 1). Whatever the roots of the protection, it is seen systemically with the major organs, heart, liver, and kidney all displaying the phenotype.

Tissue Specific Ischemia/Reperfusion Injury Protection

During the hibernating season there is a lack of overt tissue damage to brain tissue, cardiac muscle, kidney, or liver either during torpor when animals are well oxygenated or upon arousal when the animal is in a hypoxic transition to euthermia.

Brain

The brain of a hibernator tolerates pronounced fluctuations in blood flow during torpor and arousal cycles. Hibernation is neuroprotective, with many of the putative protective mechanisms mirroring those that prevent MOF, including hypothermia, antioxidant defenses, immune system modulation, and metabolic suppression (reviewed in Drew et al. 2001)(176).

The parameters affecting resistance to brain injury in ground squirrels seems to depend on intrinsic properties of the neurons that vary between species and, in some cases, between seasons. Hippocampal slices from active and hibernating ground squirrels (*Citellus tridecemlineatus*) withstand oxygen-glucose deprivation (OGD, an *in vitro* ischemia/reperfusion model) at low temperatures (20 and 7°C). However, at euthermic temperatures (36°C) the slices from the summer animals fared no better than those from non-hibernating, control species (84). Interestingly, AGS have shown to be resistant to neuronal damage from hypoxia and ischemia, regardless of hibernation season. Although P_{aO_2} and hemoglobin saturation both drop during arousal in the AGS, there is no indication of cellular stress or pathology in several brain regions (83, 158). Additionally, hippocampal slices from torpid and IBA-AGS showed intrinsic tissue properties that allow them to retain their ability to tolerate OGD (190). Notably, euthermic AGS are also protected from neuronal death in the hippocampus after asphyxia CA (global cerebral

ischemia), which may stem from a season-independent ability to maintain neuronal ion homeostasis (171, 191).

Heart

Even at low body temperature, hibernators are able to regulate the contractile function of their cardiac muscle. The molecular basis for this regulation is unknown. However, there are some molecular differences in the hibernators' heart that may contribute to its continued functionality as well as its protection from I/R injury.

The hibernator's heart has a superior ability to regulate Ca^{2+} concentrations. In two hibernating ground squirrel species, there is a novel isoform of Ca^{2+} binding protein calsequestrin that is expressed in their cardiac sarcoplasmic reticulum (SR)(192). SR activity is seasonally dependent and at its highest levels during torpor. SERCA2a, sarco(endo)plasmic reticulum Ca^{2+} -ATPase 2a, expression is increased during hibernation. Higher SERCA2a density in the SR allows for an increase in Ca^{2+} uptake in addition to ATP hydrolysis. High rates of ATP hydrolysis may provide regional endothermy within cardiomyocytes despite a low body temperature (5-6°C)(99). Additionally, a modified ryanodine/ Ca^{2+} channel found in the heart of hibernators increases Ca^{2+} up take from the cytoplasm (compared to rats) over a temperature range from 5 to 37°C (193). Combined, these modifications enhance the ability of hibernators to regulate Ca^{2+} uptake/storage/release from the SR in cardiac muscle at low body temperatures.

There is also a metabolic fuel switch in the heart during torpor. Heart PDK4 (pyruvate dehydrogenase kinase 4) is upregulated in the heart during hibernation (194). PDK4 inactivates pyruvate dehydrogenase, thereby blocking the conversion of pyruvate into acetyl-CoA. In doing so, glycolytic intermediates are blocked from entering the TCA cycle. PDK4 is the isoform least sensitive to inhibition by pyruvate accumulation (194). Using C^{14} -labeled metabolites with NMR, it was found that the ketone body B-hydroxybutyrate (DBHB) is preferentially used in the heart (and brain) as a fuel source during torpor (99). There is a 15-fold increase in circulating ketone bodies

during hibernation (78). DBHB has been found to be beneficial in extending the survival of ground squirrels (and rats) subjected to acute hypoxia. In addition, when administered with melatonin, DBHB increases the survival time of rats during hemorrhagic shock (91). Upregulation of gap junction protein production and an increase in gap junctions during cold adaptation in hibernators may provide low-resistance intracellular channels, thereby maintaining synchronous contraction (195, 196).

Liver

Studies of protective mechanisms in the livers of obligatory hibernators have uncovered differences in mitochondrial function during hibernation. These changes support the idea of rapid suppression of metabolism during entrance into torpor and the reversal of metabolic suppression upon arousal (slow in mitochondria but rapid on the whole-animal level) (197). Specifically, the mitochondria state 3 (fully coupled) respiration decreased in the livers of torpid hibernators. This depression is reversed upon each interbout arousal. The depression did not occur until after the animals were torpid and was not reversed until after the animals began to arouse. There were no differences in the proton leak (state 4) among the hibernation stages. There was a reduction in state 4 respiration but it was due to a decrease in the reactions used to generate the proton gradient and not to a change in proton permeability of the mitochondrial membranes (72).

After prolonged (72 hr) cold ischemia and reperfusion, livers from hibernators showed fewer signs of injury than livers from summer squirrels or rats. Hibernators' livers had a greater ability to maintain mitochondrial respiration, sinusoidal lining cell viability, and bile production, while having lower vascular resistance and phagocytosis of Kupffer cells. The protection did not depend on whether animals were torpid or not during I/R (102).

Kidney

It has been found that kidneys from both summer- and winter- season thirteen lined ground squirrels were protected from I/R necrosis and apoptosis *ex vivo* (198). However, IBA

kidneys incurred brush border injury that was not present in kidneys from torpid animals after the same insult. Notably, these experiments were conducted under cold ischemia (4°C) and warm reperfusion (37°C) where the decreased temperature may have augmented innate protection. A separate study found an increase in Cox 1 (cytochrome c oxidase subunit 1) in kidneys during hibernation. It is hypothesized that this may minimize damage to the electron transport chain during periods of low T_b and/or ischemia/reperfusion (199).

Conclusion

Ischemia/reperfusion injury and the subsequent development of multi organ dysfunction syndrome or MOF poses a serious medical complication to trauma patients. Obligate hibernators such as ground squirrels may hold insight into possible interventions to ameliorate the downstream organ damage from I/R injury. To date, researchers have explored several of the mechanisms that may contribute to the ability of these animals to repeatedly tolerate I/R, including metabolic intermediates, antioxidants, hypothermia, immune system alterations, and the ability of the small intestine to withstand I/R. Although each may contribute to a hibernators ability to rewarm from the torpid state without incurring tissue damage, there is no consensus on what role these factors play in the documented ability of these animals to tolerate trauma-induced I/R. More likely than not, there is not a single mechanism of injury avoidance, but a network of redundant systems. This insures that these animals are able to survive and thrive through multiple iterations of suppressed blood flow followed by rapid return of normal circulation over the course of a hibernation season or during a more human-paralleled event such as CA or HS.

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Tables and Figures

Table 1.1. Summary of potential protective mechanisms employed by ground squirrels against ischemia/reperfusion injury.

Mechanism	Possible contribution to protection from I/R
Metabolic Switch (from carbohydrate- to lipid-based metabolism)	<ul style="list-style-type: none"> • no increase in lactate during torpor via decreased production and/or increased utilization • higher DBHB during hibernation: unknown mode of protection, greater energy per unit than pyruvate • PPAR activation by FFA: increase in fatty acid metabolism, decrease in glucose metabolism, decrease in inflammatory gene expression, increase in UCP-1 expression
Antioxidants	<ul style="list-style-type: none"> • ascorbate, melatonin, SODs, and catalase
Hypothermia	<ul style="list-style-type: none"> • reduce reaction rates that could form ROS
Depressed Immune Function	<ul style="list-style-type: none"> • 100-fold drop in circulating leukocytes during torpor
Small Intestine Protection	<ul style="list-style-type: none"> • no damage to small intestine after I/R in IBA animals: increase in HMGCS2, albumin, greater IgA

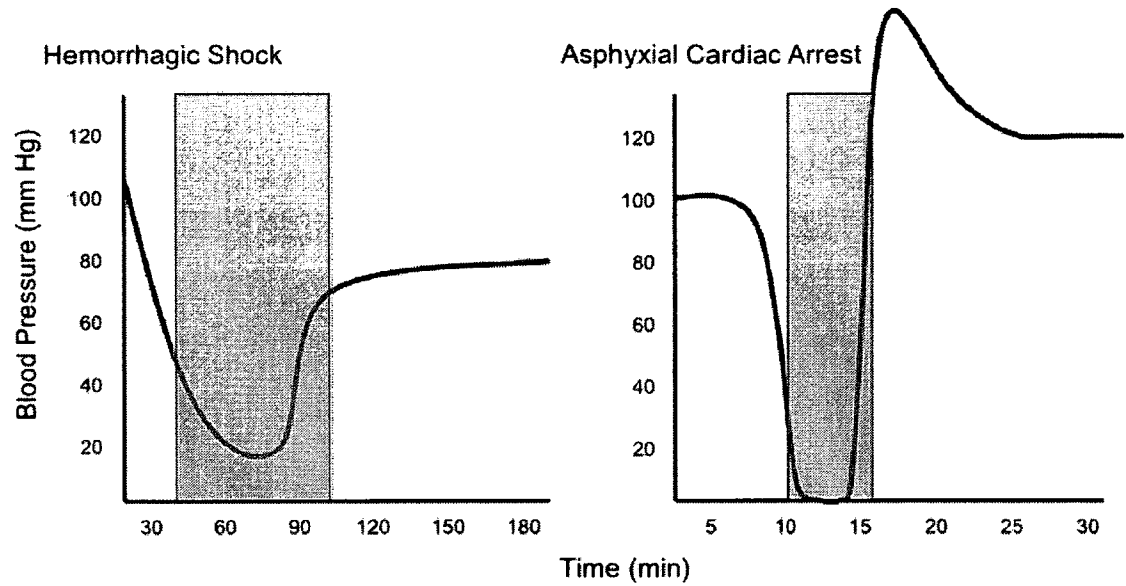


Figure 1.1. Hemorrhagic shock and asphyxia cardiac arrest are two models of global ischemia/reperfusion injury that can lead to multi organ failure. Grey bars represent period of ischemia and subsequent reperfusion.

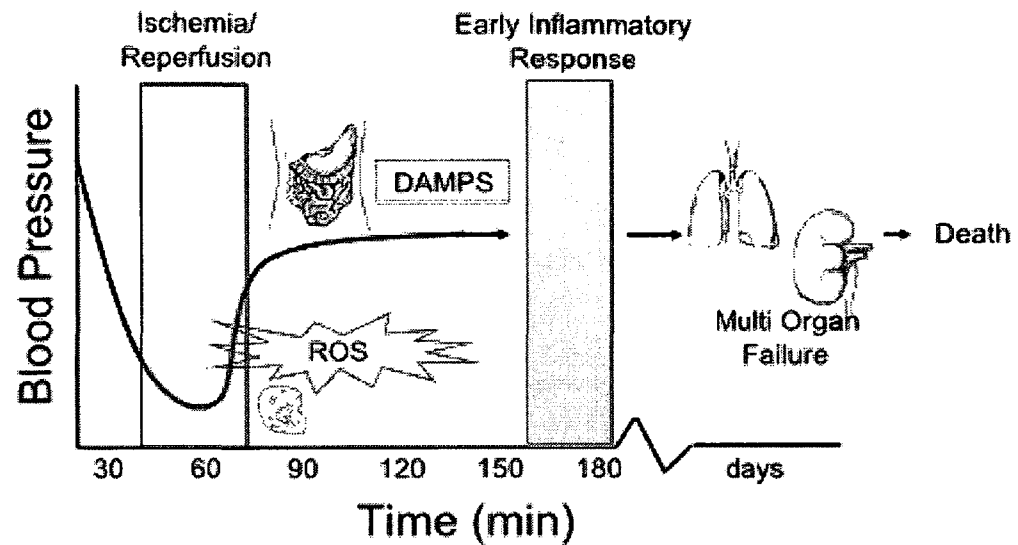


Figure 1.2. Timeline for events in the current theory of small intestine-mediated progression of global I/R injury to multi organ failure and death. Damage to the small intestine during I/R by ROS induces the formation and subsequent release of danger associated molecular patterns (DAMPS) into circulation. DAMPS provoke an early unregulated systemic inflammatory response that then damages other organs leading to their failure and death of the individual.

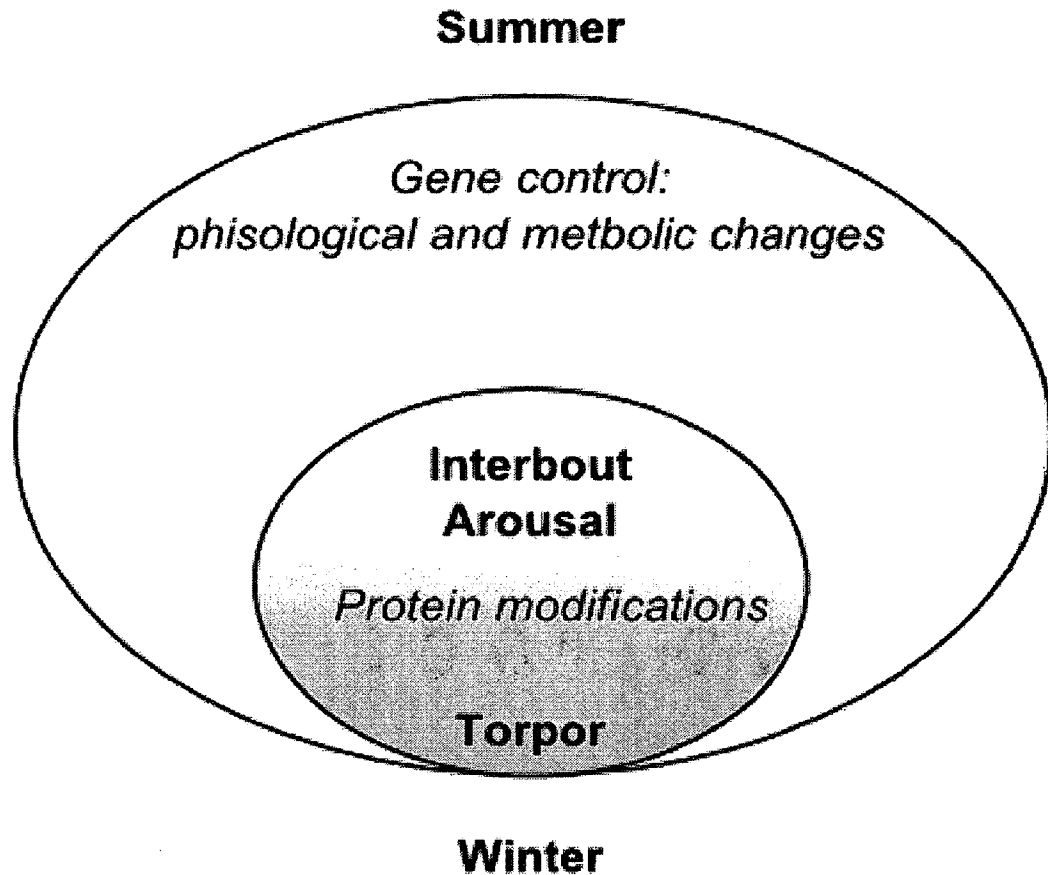


Figure 1.3. The two switch model is used to describe the physiological and metabolic states of hibernating ground squirrels. AGS have a circannual rhythm consisting of summer/active and winter/hibernation seasons. Only during the winter/hibernation season can AGS enter a torpid state. The first switch that occurs during the summer /winter transitions consists of metabolic and physiological alterations regulated by gene control. The second switch, which occurs between torpor and interbout arousals during the winter season, is controlled through modifications of regulatory proteins.

Chapter 2. Resistance to multi organ damage after global ischemia/reperfusion in the arctic ground squirrel¹

Abstract

Objective: Cardiac arrest and hemorrhagic shock are two clinically relevant situations where the body undergoes global ischemia as blood pressure drops below the threshold at which tissues can be adequately perfused. Resistance to ischemia/reperfusion (I/R) injury is a characteristic of hibernating mammals. The present study sought to determine if arctic ground squirrels (AGS) are protected from multi organ failure after cardiac arrest- or hemorrhagic shock-induced global I/R and if protection is dependent upon their hibernation season.

Design: Non-randomized controlled trial.

Setting: Laboratory.

Subjects: Sprague-Dawley rats, euthermic AGS, and interbout arousal AGS.

Interventions: Organism's innate resistance to I/R injury.

Measurements and Main Results: For cardiac arrest, rats and euthermic AGS were asphyxiated for 8 min, inducing cardiac arrest. For hemorrhagic shock, rats, euthermic AGS, and interbout arousal AGS were subject to HS by withdrawing blood to a MAP of 35 mm Hg. Low MAP was maintained for 20 min and then the animals were reperfused. For both I/R models, the animals' temperature was maintained at 36.5-37.5°C. After reperfusion, animals were monitored for seven days (CA) or 3 hrs (HS) then tissues and blood were collected for histopathology, clinical chemistries, and cytokine level analysis (HS only). For the HS studies, a subset of rats and AGS were monitored for three days after HS to assess survival and physiological impairment.

¹ Bogren LK, Carpluk J, Drew KL, prepared for submission to Critical Care Medicine, 2013.

Regardless of season AGS showed no physiological deficit 12 hours after HS. Blood chemistries indicated no organ damage in the ground squirrel while the rat showed signs of liver damage. In addition, AGS had a lower cytokine response after HS than did rats.

Conclusions: AGS are resistant to organ damage, systemic inflammation, and multi organ failure after I/R and this resistance is not dependent on their ability to become hypothermic during insult but may stem from an altered metabolic response during I/R.

Introduction

Multiple organ failure (MOF) after trauma and/or hypoperfusion, such as occurs during systemic ischemia/reperfusion (I/R) injury, has a mortality rate between 30-100%. Currently there is no treatment available that can halt or reverse its progression (1). Two perpetrators of systemic I/R injury are cardiac arrest (CA) and hemorrhagic shock (HS). During both cardiac arrest and hemorrhagic shock, the body undergoes global ischemia as blood pressure drops below the threshold at which tissues can be adequately perfused. This results not only in a shortage of oxygen, but also a lack of energy substrates and the removal of harmful waste products. If the ischemia persists, ATP levels in a cell can be depleted resulting in the inability to maintain membrane integrity and ion gradients, eventually leading to cell death (2, 3).

Reintroduction of oxygen during reperfusion also causes damage via the formation of reactive oxygen species and subsequent oxidative stress (4). Some studies support a role for lactate and acid base balance as early indicators of oxygen debt and potential modulators of inflammation (5-8). Other studies suggest that injury to the small intestine initiates an unregulated systemic inflammatory response that propagates MOF (9-14). Study of a novel species that resists I/R and MOF may lead to a better understanding of the early events leading to MOF and may identify innovative therapeutics.

Humans and most other mammals are vulnerable to I/R injury and MOF. However, hibernating mammals have been shown to be resistant to I/R injury in numerous isolated organ preparations. In particular, ground squirrels (GS) have been found to be protected against intestinal I/R injury during winter season (15, 16). Livers from winter-season GS were shown to be resistant to cold I/R injury (17) while kidneys from summer- and winter season GS were protected from cold ischemia /warm reperfusion necrosis and apoptosis ex vivo (17, 18). In addition, hippocampal slices from interbout arousal (IBA) GS were resistant to I/R (19) and hippocampus and striatum in vivo were protected after global ischemia (cardiac arrest) in arctic ground squirrels (AGS, *Urocitellus parryi*) (19, 20). Previous studies have not investigated if GS are protected from MOF after global I/R, and if the animals are resistant to injury, where in the early pathogenesis of MOF do GS differ from I/R injury prone animals?

Here, we test the hypothesis that AGS will be protected from MOF after global I/R injury. We further proposed that protection levels may vary seasonally (summer active versus winter hibernation) as there are physiological and metabolic alterations that protect the animals from I/R experienced during the periodic arousals from torpor experienced during the hibernation season. Alternatively, since AGS brains resists I/R injury in both the summer and winter season (21, 22), hibernation season may not play an essential role in I/R tolerance in this species of ground squirrel. We first determined if AGS were protected from MOF after I/R in two clinically-relevant models, cardiac arrest and hemorrhagic shock. Next, we utilized the HS model to further delineate probable avenues for protection.

We found that the AGS were protected from MOF after both CA and HS and that protection was independent of season; the response was similar in summer, euthermic (EU) animals and winter, IBA animals. After HS, AGS maintained a positive base excess (BE), even up to three hours after reperfusion. Additionally, their small intestine was not damaged, nor did they have increased levels of circulating cytokines. Overall, the results indicate that AGS

protection from I/R injury and MOF stems from an altered lactate and base excess response to I/R.

Materials and Methods

CA and HS were studied as clinically relevant models of I/R. HS was examined in summer and winter season to determine if hibernation phenotype plays a role in the response of AGS to I/R.

Animals

AGS were lived trapped in Northern Alaska (66°38'N, 149°38'W) as juveniles and were at least one year of age at the time of the experiment. Once at UAF, they were housed at 17°C under light conditions based on 69° latitude. Cardiac arrest animals were maintained at those conditions and experiments conducted in June and July. For hemorrhagic shock, in late August, AGS were moved to a cold room (2°C) and kept on a 4-hour light: 20-hour dark cycle. Food and water was available ad libitum until 20 hours prior to surgery at which time the food was removed. Female and male AGS (438-1123g) were used in this study due to the availability of wild-caught animals. All summer/euthermic animals were in their post-reproductive/ summer active phase as evidenced by body temperature, activity, and lack of spontaneous torpor for at least four weeks. Winter/hibernating animals were distinguished by having regular spontaneous torpor bouts for at least eight weeks prior to the experiments. Supplemental Tables 1-5 delineate characteristics used to assess hibernation season. Male Sprague-Dawley rats (250-320g for CA and 350-420g for HS) were used as positive, I/R sensitive controls. Rats were purchased from Simpson Laboratory (Gilroy, CA) or from UAF Animal Resource Center (Sprague-Dawley, Simonson Laboratory, Gilroy, CA derived rats). All animal procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the Animal Use and Care Committee of the University of Alaska Fairbanks (UAF) with the exception of two instances. In these cases, pharmaceutical-grade Ringers was not available and Ringers solution was prepared

in accordance with the Guide, but without knowledge that use of non-pharmaceutical grade solutions required IACUC approval.

Cardiac Arrest

The asphyxial CA model was employed as described previously (20, 23) except as noted. Positive end-expiratory pressure (PEEP; 1 cm H₂O) was maintained throughout manual ventilation and baroreceptors along the carotid artery were stimulated by gentle pressure on the neck to increase mean arterial pressure (MAP) to ~100 mm Hg in the AGS prior to asphyxia. The femoral cannula was externalized at the base of the neck. MAP was calculated as 2/3 (end systolic pressure) x 1/3 (end diastolic pressure). Blood samples for serum and EDTA-plasma were collected from the femoral artery immediately prior to CA (baseline) and 24 hours after return of spontaneous circulation (ROSC), and via cardiac puncture seven days after CA (Fig. 2.1). Sham animals underwent the cardiac arrest surgery but were not disconnected from the ventilator to produce asphyxia.

Hemorrhagic Shock

Surgical preparation, cannulation, and monitoring of the animals were the same as for the CA procedure except that both femoral arteries were cannulated and the right femoral artery was not externalized. After cannulation and the stabilization of blood gases, the animals were subjected to hemorrhage over a 15 minute period that resulted in a decrease in MAP to ~35 mm Hg. MAP was held at that level for 20 minutes before animals were then given intravenous non-lactated Ringers solution equal to 2/3 volume of the blood removed during hemorrhage. Reperfusion occurred over a period of 15 minutes at a constant infusion rate. After reperfusion, animals were monitored for three hours. Blood samples were collected from the femoral artery immediately prior to HS (baseline), upon end of reperfusion, and from cardiac puncture at the end of the three hour monitoring period (Fig. 2.1). Sham animals were subject to the hemorrhagic

shock procedure, but blood was not removed and Ringers was not administered. For survival assessment, animals (n=4 per group) were placed back into cages after the three hour monitoring period and observed for physiological deficits for up to 72 hours. In the isovolumetric studies, animals (n=4 per group) had 55% of their calculated total blood volume removed during hemorrhage, regardless of the resultant MAP. Total blood volume was calculated as being equal to 6.5% of the animal's total body weight.

Non-lactated Ringers solution (148 mM NaCl, 2 mM CaCl₂, 4 mM KCl) was obtained commercially (Hospira, Inc., Lake Forest, IL) for all procedures save two, where it was unavailable. In these instances, Ringers was made with sterile water for injection (Butler & Schein, Columbus, OH) and sterile filtered through a 0.2 µm syringe filter (HT Tuffryn Membrane; PALL Life Sciences) prior to use.

Blood Chemistries and Cytokine Levels

For glucose and lactate, blood was collected in EDTA and the plasma analyzed with a YSI 2300 STAT glucose/L-lactate analyzer (Yellow Springs, OH). Unlactated Ringers was used as the infusion solution to eliminate external sources of lactate. Analysis of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine phosphokinase (CPK), and creatinine was conducted on serum with a Vitros 5600 (Ortho Clinical Diagnostics, Rochester, NY) using standard methods. Base excess, bicarbonate (HCO₃⁻), pH, P_{CO2}, and P_{O2} levels were ascertained with i-STAT CG8+ cartridges (Abbot, Princeton, NJ) using whole blood. Cytokine levels in EDTA-plasma (snap frozen in liquid nitrogen after collection and stored at -80°C) were determined using the Rat Cytokine 10-Plex Panel (no. LRC0002) from Invitrogen (Grand Island, NY) on the Luminex 200 System (Austin, TX).

Quantitative histological analysis

Immediately following euthanasia, tissues were collected and suspended in 10% buffered formalin and sent to VDX Pathology (Davis, CA) for trimming and processing into histological

slides for evaluation by a veterinary pathologist. At VDx, the tissues were examined and graded according to a standard format based on a dictionary of changes specific for each tissue. The subjective evaluations of change in each tissue were converted into system of numerical evaluation that provided 16 levels of injury for each type of change evaluated for the extent of lesion size and for degree of tissue change. These data were then summarized for each tissue to provide a severity summary that permitted comparison of changes between species.

Morphological analysis of small intestine

Small intestine was formalin fixed, blocked in paraffin, sectioned and stained with hematoxylin and eosin. From each slide, five fields with a total of ~50 villi from each animal were analyzed using light microscopy at 2-10x in a blinded fashion. The length of each villi was measured using MetaMorph (Molecular Devices, Sunnyvale, CA) and was assessed a damage score as previously described (24). Briefly, 0 = normal villi, 1 = mild damage with vacuolation at villus tip, 2 = increased space at tip, lifting of epithelial layer from the lamina propia, 3 = moderate damage with massive lifting of subepithelium and vacuolation to the midpoint of the villi, 4 = vacuolation to the base of the villi, 5 = severe damage with mucosal ulceration and loss of villi structure.

Statistics

All data is expressed as mean \pm S.E.M. Statistical analysis was performed using SAS (Cary, NC) Physiological parameters were analyzed via ANOVA with repeated measures. Blood chemistries (AST, ALT, CPK, and creatinine) and plasma cytokine levels were evaluated with ANOVA and small intestine morphological analysis with a two-way ANOVA. Finally, glucose, lactate, and BE for HS were evaluated by two-way ANOVA with repeated measures. All significant effects in ANOVAs were followed by a Tukey's post-hoc test. Statistical significance was considered to be a p value of <0.05 .

Results

Cardiac Arrest

To test the hypothesis that AGS resist MOF after I/R, we first determined if the severity of ischemia during asphyxia-induced CA was similar between rats and AGS. Mean arterial pressure (MAP) decreased to <20 mm Hg for both species after 3.5 minutes of asphyxiation and remained under 20 mm Hg for the duration of the arrest (Fig. 2.2). After return of spontaneous circulation, MAP increased in both species. The AGS had a significant increase in MAP immediately following the onset of asphyxia compared to the rats ($p=0.0119$, ANOVA, time x species). Despite the increase in MAP at the start of asphyxiation, the AGS had similar MAPs for the remainder of the asphyxiation/CA period except for the 5 minute time point. After onset of asphyxia, functional heart beat, as determined by a plusatile increase in MAP, became uncoupled from cardiac contraction noted on the ECG. Functional HR was therefore determined from the transient increases in MAP due to pulse pressure. Heart rate (HR) for AGS and rats declined in a similar manner after onset of asphyxia (Fig. 2.2) however, HR persisted in AGS more so than rat ($p<0.0001$, ANOVA, time x species). By 6.5 minutes into asphyxiation, four out of five rats had a HR of 0 whereas only one AGS had a HR of 0 at 8 minutes. HR in the other AGS remained above 10 bpm throughout the period of asphyxia. Both AGS and rats regained pre-CA HR by one hour after CA.

We next assessed if recovery of basic physiological parameters following ROSC were similar between rats and AGS. There were no statistically significant differences in whole blood pH, P_{CO_2} , P_{O_2} , HCO_3^- , or BE before and after ischemia in AGS or rats as shown in Table 2.1. AGS had a higher BE than rats before and after CA ($p=0.0486$, ANOVA, main effect of species).

Lastly for the CA experiments, we sought to determine if organ damage after CA was comparable in the rats versus AGS. Serum markers showed evidence of organ damage after CA in rat but not in AGS (Fig. 2.3). Plasma levels of both ALT and AST, markers of liver damage,

were increased one hour after ROSC in the rats as compared to shams ($p=0.0002$, ANOVA, group x species x time). AGS did not show liver damage after ROSC as indicated by no significant change in ALT and AST levels. AGS and rats both tended to have an increase in creatine phosphokinase 24 hours after CA which is consistent with heart injury. Lactate dehydrogenase also tended to increase after CA in the rats; indicative of systemic cellular damage. However, due to technical problems, samples size was insufficient for statistical analysis for these two parameters (Supp. Fig. 2.1).

Histopathological analysis of the livers showed multifocal ischemic necrosis in the rat after CA but not the AGS (Supp. Fig. 2.2). In the rats, accentuation of zonation was found to be present in some animals. Few changes were found in this area in the AGS while changes of this type were more frequent in the CA rats. Several rats had areas of infarction or necrosis in the liver, with no changes in this type found in the ground squirrels.

Quantitative histopathological analysis of the other organs did not reveal significant organ damage and/or failure in either the rats or AGS in CA versus sham animals. Heart, lung, spleen, skeletal muscle (right soleus), pancreas, kidney, stomach, small intestine, and large intestine were all examined for signs of damage. No changes following I/R were identified in these tissues in either species.

Due to the mild degree of organ damage seen after CA in the rat, a more severe model of I/R, hemorrhagic shock, was employed. In addition, instead of allowing the animals to recover for seven days and possibly repair any ischemic damage, tissues were collected three hours after I/R in the hemorrhagic shock model. We also assessed response to HS in the AGS during the summer and winter seasons.

Hemorrhagic Shock

For the HS model, the responses of both summer season (euthermic; EU) and winter season (interbout arousal; IBA) squirrels to hemorrhage and reperfusion were compared.

Euthermic AGS had not experienced a spontaneous bout of torpor for at least four weeks, whereas IBA animals had displayed two to five months of spontaneous torpor prior to HS (Supp. Table 2.2). At the outset of the experiment, the IBA animals were still alert, responsive and at euthermic core T_b ($\sim 35^\circ\text{C}$) and HR (~ 230 - 270 BPM).

To first determine if HS was a severe model of I/R, survival was assessed in rats and AGS for up to 72 hours after HS. All of the animals had similar total blood volume removed as a percent of body weight to achieve a MAP of 35 mm Hg (AGS-EU: $31.1\% \pm 3.5$, AGS-IBA: $29.4\% \pm 7.3$, Rat: $35.1\% \pm 3.4$). We found that AGS, regardless of hibernation season, survived for the 72 hour monitoring period after HS with no observable physiological deficit whereas rats did not survive overnight (Fig. 2.4). Three hours post- resuscitation, the AGS were lethargic, but responsive to stimuli. The rats were unresponsive at this timepoint (Supp. Data). Eighteen hours after HS, the AGS had resumed normal appearance and behavior. None of the rats survived to 18 hours post-HS.

As the AGS were able to survive a $\sim 30\%$ blood loss, their ability to survive a greater degree of hemorrhage was examined. Both euthermic and IBA AGS were subject to a $\sim 55\%$ blood loss (AGS-EU: $56.9\% \pm 1.8$; AGS-IBA: $56.0\% \pm 0.8$; Fig. 2.4). The IBA animals survived ~ 1 hour after infusion, but none survived three hours post resuscitation. The euthermic AGS all died within 30 min of resuscitation except for one animal who survived the entire three hour monitoring period.

For the HS model, we assessed if the animals received similar severity of hemorrhage-induced ischemia. During the hemorrhagic shock experiments, MAP for all animals was brought to 35 mm Hg during hemorrhage and held there for 20 minutes before animals were resuscitated with Ringers solution. Rats had a higher initial MAP than did the AGS ($p=0.0027$, ANOVA, effect of time x group; Fig. 2.5). However, percent blood removed, as estimated from body mass, was not affected by initial MAP (AGS-IBA: $33.11 \pm 5.39\%$, AGS-EU: $36.36 \pm 4.86\%$, rat: $34.85 \pm 2.01\%$).

All three hemorrhaged groups had different MAPs at the end of infusion with rat being the highest (65.00 ± 2.24 mm Hg) followed by AGS-IBA (47.14 ± 2.14 mm Hg) then AGS-EU as the lowest (38.33 ± 1.54 mm Hg; $p < 0.05$ Tukey). Rat MAP increased initially but was not sustained. AGS-IBA MAP slowly increased to pre-HS values while AGS-EU did not return to pre-HS levels. MAP in all groups was similarly low 3 hours post-HS. In contrast to MAP, HR remained constant during hemorrhage and reperfusion for the rats but increased at the end of HS in AGS ($p = 0.0061$, ANOVA, effect of time x group; Fig. 2.6). Both AGS groups had a lower HR at the start of the experiment and at the end of infusion than the rats ($p < 0.05$, Tukey). Rats had a higher HR than the IBA AGS at the start of withdrawal ($p < 0.05$, Tukey) and from the EU AGS at the end of withdrawal and three hours post HS ($p < 0.05$, Tukey). IBA AGS and EU AGS were not statistically different at any timepoint measured during the experiment ($p \geq 0.05$, Tukey).

Next we asked if hemorrhage and reperfusion had a differential effect on systemic physiology between IBA AGS, euthermic AGS, and/or rats by monitoring several physiological parameters at the start of hemorrhage (baseline), end of reperfusion, and three hours after I/R (Fig. 2.1). Table 2.2 lists the physiological parameters and the corresponding values obtained. Physiological parameters, except for P_{CO_2} , changed for all groups during hemorrhage and resuscitation. All groups showed a decrease in pH over time ($p < 0.0001$, ANOVA, main effect of time). AGS (IBA and EU) had a higher pH than rat ($p = 0.0009$, ANOVA, main effect of group). Base excess also decreased over time ($p < 0.001$, ANOVA, main effect of time) with AGS having a higher BE than rats ($p = 0.0003$, ANOVA, main effect of group). Bicarbonate levels decreased over time for all groups ($p < 0.0001$, ANOVA, main effect of time). P_{CO_2} and P_{O_2} remained unchanged over time with rats having a lower P_{O_2} than either group of AGS ($p < 0.001$, ANOVA, main effect of group). AGS-EU had a lower initial blood glucose than did rats ($p < 0.001$, ANOVA, effect of time x group). AGS-IBA had higher initial blood glucose than AGS-EU and higher blood glucose than both AGS-EU and rats at the end of reperfusion and three hours afterwards

($p < 0.001$, ANOVA, effect of time x group) despite all groups being fasted ~20 hours prior to surgery.

Blood markers to assess organ damage showed less organ damage in AGS than in rat. Rats had a significant increase in serum creatinine, a marker for kidney damage, after HS as compared to baseline ($p < 0.001$, ANOVA, main effect of group). Neither IBA nor euthermic AGS had significantly elevated plasma levels of creatinine as compared to baseline after HS (Fig. 2.7). Rats tended to show elevated levels above baseline of ALT and AST compared to sham animals, indicating liver damage. However, none of the groups had significant increases above baseline in AST or ALT as compared to shams. Quantitative histopathological analysis of the organs showed no evidence of injury at the 3 hour timepoint. Heart, lung, liver, spleen, and kidney were examined and rated for gross structural and/ or cellular damage after HS. Overall, HS did not induce significant histopathological changes in any of the organs for any test group (Supp. Table 2.6).

As MOF is thought to originate from I/R damage to the small intestine that then instigates a systemic inflammatory response (9-14), additional measures were taken to assess intestinal injury from changes in villi length and a damage score. None of the groups showed a HS-induced change in villi length or damage score (Fig. 2.8). Rats had shorter villi than AGS ($p > 0.0001$; ANOVA, main effect of group). However, the overall villi length of any group did not decrease below 500pM. Quantitative histopathological examination of the intestine did not show any damage due to I/R with the damage score being less than 1.5, even in the hemorrhaged animals. Overall the rats, both sham HS (SHS) and HS, had a higher damage score than AGS ($p > 0.0001$; ANOVA, main effect of group).

Without substantial damage to the small intestine, we next determined if changes in serum levels of inflammatory cytokines were consistent with an inflammatory response after HS. The results demonstrate that rats displayed an inflammatory response after HS where AGS, in

either season, did not. There was a significant difference between HS vs. SHS in rats for IL-1 alpha ($p=0.0006$), IL-1 beta ($p=0.004$), TNF-alpha ($p=0.0049$), and INF gamma ($p=0.0029$, ANOVA, effects of group x treatment). Fold changes in IL-6 ($p<0.0061$) and IL-10 ($p<0.0259$, ANOVA, main effects of group) were different between AGS and rats. However, although they were not significant between SHS and HS for any group, rats showed an increasing trend after HS. By contrast, after HS-induced I/R, AGS did not demonstrate a systemic inflammatory response. Circulating levels of IL-1 alpha, IL-1 beta, IL-6, IL 10, TNF-alpha, and INF-gamma all remained unchanged after hemorrhage and reperfusion versus sham (Fig. 2.9). For some cytokines, the levels decreased after hemorrhage. This is most likely due to levels remaining constant but being diluted with hemorrhage and then subsequent reperfusion with Ringers. Total volume of Ringers returned after HS was 5.56 mL, 9.35 mL, and 8.71 mL for rat, AGS-EU, and AGS-IBA, respectively. Levels of circulating cytokines (pg/mL) in naïve animals are given in Supp. Table 2.7.

Next, we looked at possible cellular mechanisms of protection against I/R injury. Hibernators are well known for their ability to suppress their metabolism as well as their ability to switch from a glucose- to fat-based metabolism. The possibility that metabolic alterations present in the AGS may have been associated with their protection against HS-induced organ damage was investigated by monitoring glucose and lactate levels during the HS experimental protocol and afterwards. IBA AGS had increased levels of plasma glucose while rats' plasma glucose levels decreased during and after HS as compared to sham ($p<0.0001$, ANOVA, effect of group x treatment x time; Fig. 2.10). AGS-EU had no difference between HS and SHS glucose levels at any timepoint. Concurrently, lactate levels increased during and after I/R as compared to sham in both IBA AGS and the rat while not differing from sham animals for the AGS-EU group ($p=0.005$, ANOVA, effect of group x treatment x time). Lactate levels for all animals were the same at the start of hemorrhage. During hemorrhage, lactate levels rose to a greater extent in the rat (6.88 ± 0.38 mmol/L) than in the IBA AGS (4.48 ± 0.85 mmol/L). Euthermic AGS had the lowest

increase in lactate of all the test groups (3.22 ± 0.63 mmol/L). During and after hemorrhage, IBA AGS and rats had significantly elevated lactate:glucose ratio compared to sham animals while the euthermic AGS HS had ratios comparable to sham animals ($p < 0.0001$, ANOVA, effect of group x treatment x time). These results suggest a difference in metabolic response to I/R injury in the EU-AGS versus the IBA-AGS and rats.

To examine the role of acid-base balance in HS-protection, we examined BE levels over the course of hemorrhage and reperfusion. Base excess values, which correspond to the metabolic component of systemic pH levels, corroborate a metabolic component to the protection from I/R organ damage observed in the AGS. All animals initially had positive BE values (Fig. 2.11). The AGS (both seasons) had a starting BE higher than the rats ($p < 0.0001$, ANOVA, effect of treatment x time). IBA AGS had similar BE values compared to sham during and after I/R. In contrast, AGS-EU HS and rat HS had a continued decline compared to sham. From the start of hemorrhage to three hours post resuscitation, all groups had statistically the same overall decrease in BE. However, both IBA and euthermic AGS were able to maintain a positive BE for the duration of the experiment, whereas in rats, the decline resulted in a negative BE during and after hemorrhage.

Discussion

Two of the leading causes of death and disability in the US, cardiac arrest and hemorrhagic shock, involve I/R injury. Cardiac arrest represents the most severe form of I/R where blood pressure drops quickly before the heart stops, and circulation ceases then resumes with resuscitation. Worldwide, hemorrhagic shock is the number one cause of death in trauma patients, the majority of patients die from MOF (9, 25). Obligate hibernators such as ground squirrels have the unique ability to resist injury from ischemia/reperfusion. Here we used the arctic ground squirrel as a model system of I/R tolerance to probe for possible mechanisms of

protection against I/R injury and the propagation of multi organ failure. Two clinically relevant I/R injuries were investigated. Asphyxial cardiac arrest was used to determine if AGS resist MOF, while HS was used to model another clinically relevant model of I/R and to further probe the possible mechanisms of resistance.

Here we show for the first time that resistance to I/R injury in a hibernating mammal includes resistance to MOF and resistance is independent of hibernation season and hypothermia. We found that rats responded to I/R as expected of an I/R vulnerable species in a manner similar to that described for humans while AGS resisted I/R injury and MOF after both CA and HS. Classically, the first indication of the progression of I/R to MOF is an increase in circulating lactate levels and a decrease in base excess. Simultaneously, I/R damage to the mucosal layer of the small intestine induces a systemic inflammatory response that then stimulates damage to other organs, leading to their dysfunction and ultimately death (5-8).

After HS-induced I/R, rats had an increase in lactate (normalized to glucose levels) while their base excess decreased to negative values by the end of infusion and continued to decline three hours after reperfusion. Although no damage was seen in the rats' small intestine, an inflammatory response, as evidenced by the increase in plasma levels of cytokines, was generated. After I/R the rats had increased serum markers of organ damage and were unable to survive eighteen hours post I/R. Conversely, EU-AGS maintained normalized lactate levels and both IBA- and EU-AGS preserved positive BE throughout the experiment. In addition, AGS did not mount an inflammatory response and did not sustain organ damage. These findings are important because they demonstrate that the AGS are resistant to organ damage after I/R in a euthermic state and that the resistance was not due to more downstream effectors of MOF such as damage to the small intestine and systemic inflammation, but most likely in a metabolic or acid/base balance adaptation that is present and evocable throughout the year.

AGS had no organ damage after CA

AGS had similar responses and duration of CA I/R insult as did the rat controls but were found to have no organ damage after CA. To first determine if AGS were resistant to MOF after I/R, only summer euthermic AGS were used, thereby eliminating the seasonality variable and providing the most theoretically I/R intolerant system for the hibernating mammal. Rats had a higher initial resting HR than did the AGS. Although the heart rate of the AGS did not cease as did the rats and was significantly higher than the rats for the last two minutes of CA, both groups reached the same MAP of ~ 0 for the last three minutes of asphyxiation. The AGS did have an increase in MAP during the first minutes of asphyxia, but by the second minute of asphyxia, AGS MAP was the same as rats. This increase was not seen in previous studies (20) and could be due to basal MAP manipulations in AGS prior to initiation of asphyxia. This may have had an effect that extended briefly into the asphyxia period. Overall, the MAP of the AGS and rats fell rapidly to <20 mm Hg after initiation of the insult.

Rats had an increase in AST and ALT, serum markers for liver damage, 24 hours after CA. Although AGS had a similar duration and extent of low MAP as the rats, they did not show increased serum markers of liver damage. Both of these are enzymes located in liver parenchyma cells and serum levels are raised when liver cells are damaged (26). AST is also present in red blood cells and skeletal and cardiac muscle and is therefore not as specific for liver damage as ALT. AST and ALT were both increased to a similar extent in the rats after CA indicating that damage had been incurred by liver cells. Results from the HS I/R experiments corroborate that the lack of liver damage in the AGS after CA was most likely due to an intrinsic lack of vulnerability to I/R damage present in the AGS rather than a difference in the severity of I/R. In the HS experiments, AGS and rats experienced the same extent and duration of I/R (Fig. 2.5), but AGS also did not show an increase in serum markers for liver damage while the rats did.

In addition to an increase in serum markers for liver damage, rats subject to CA I/R also displayed multifocal ischemic necrosis in the liver upon histopathological examination. AGS

showed no significant organ damage, in liver or other organs, upon histological examination seven days after CA. Dave et al. (2006) demonstrated that AGS were resistant to neuronal damage after CA. In that study, the MAP of the AGS decreased to 0 mm Hg by the eight minutes of asphyxia and was not statistically different from rats for the last five minutes of asphyxia. In the present study, AGS MAP also reached 0 mm Hg by eight minutes of asphyxia, but declined more slowly than MAP in rat. It may be that in this study, the degree of I/R injury was not as great as previous studies and that this may account for the lack of histological organ damage seen. Additionally, the lack of organ damage could be due to repair mechanisms employed during the seven days between CA and tissue harvest. However, in the HS model, the tissues were harvested three hours after I/R and no damage was observed at that timepoint. It could be that the HS timepoint was too early to detect damage due to apoptosis. However, as the animals survived with no apparent physiological damage for three days after HS-induced I/R, it is unlikely that gross damage to any organ did occur.

Previous studies of CA in the AGS were focused on neuronal damage after global cerebral ischemia and found that the AGS were resistant to such insult (20, 23). The current study extends those results to include the other major organs being protected from CA I/R.

Acid-base component to I/R protection

Hemorrhagic shock was used as a second clinically relevant form of I/R injury to assess the possible mechanisms of resistance against I/R- induced MOF observed in the AGS after CA. In the HS experiments, we first looked at markers of global cellular damage. If cells resist I/R damage, there would be no inflammatory response and no MOF. One possible means of cellular protection is to reduce acidosis caused by lactate generation under anaerobic metabolism. Here we found that rats had an increase in lactate levels during and after HS, as did IBA AGS. Euthermic AGS did not have an increase in lactate levels during or after HS. Additionally, the rats had marked decrease in glucose levels over the same time period while the euthermic AGS maintained glucose levels and IBA AGS increased glucose levels. Overall this indicates that the

rats are utilizing their glucose stores and processing the energy source under anaerobic conditions to produce lactate. The IBA AGS appear to be mobilizing or synthesizing glucose and metabolizing it, at least in part, under anaerobic conditions. In contrast, the euthermic AGS do not have an increase in circulating glucose levels which may indicate that they have reduced their metabolic demand and are not metabolizing glucose either aerobically or anaerobically (as there is also no increase in lactate levels). It could also be indicative of a switch in energy substrate from glucose to ones that cannot be metabolized into lactate, such as D- β -hydroxybutyrate and free fatty acids. Although more experiments are needed, the results from the current experiments suggest that, at least in part, euthermic AGS may also be able to readily utilize energy sources other than glucose such as free fatty acids and/or D- β -hydroxybutyrate during I/R and/or may also be able to reduce their metabolism.(27, 28)

Base excess was also monitored during the experiments. BE accounts for the metabolic component of acidosis. When BE is negative, it indicates that the organism is acidic and that a portion of this acidosis has a metabolic basis (e.g. lactic acid build up). Here, BE in hemorrhaged rats is negative by the end of infusion and continues to drop until three hours after infusion. In contrast, both IBA and EU AGS maintain a large positive BE over the course of the experiment. Both the AGS and rats have a decrease in BE over the course of the experiment; however, the AGS start with such a large positive BE that they are able to maintain a positive BE throughout I/R and afterwards. Taken in conjunction with the lactate and glucose data, this implies that AGS may possess a better buffering capacity that enables them to remain in acid-base balance.

AGS small intestine remained undamaged with no subsequent systemic inflammation after HS

The next step in the etiology of MOF after I/R to be examined was injury to the small intestine. MOF after HS has been shown to stem from a systemic inflammatory response to I/R damage to the small intestine that induces the release of non-microbial, inflammation-inducing “danger” molecules (e.g. danger associated molecular pattern molecules; DAMPS) into circulation (13), (14) Here, there was no histopathological damage to the small intestine after HS in the rats

or AGS. Although there was no damage seen in the small intestine of the rats after HS, there was a significant increase in the level of circulating cytokines, indicating a systemic inflammatory response. Whereas in the AGS, there was no significant change in circulating cytokine levels animals during either season. The lack of injury in the small intestine may be due to the timepoint at which it was harvested and the means used to assess damage. As the tissue was collected three hours after reperfusion, there may not have been sufficient time for necrotic and/or apoptotic process to be visible upon histopathological examination. This is unlikely as small intestine is one of the organs most sensitive to I/R where even a delay in preservation after euthanasia can produce visible tissue damage (VDx Pathology, personal communication). The preservation of the small intestine in euthermic AGS is contrary to previous studies where I/R produced extensive cellular necrosis and damage to the villi in the mucosal layer (16).

No organ damage after HS in the AGS

Subsequently we addressed if AGS incurred organ damage after HS-induced I/R. In MOF organ damage occurs to organs that were not directly subject to injury but who are later damaged due to a systemic inflammatory process sparked from the initial injury. Here, serum markers for liver (AST and ALT) and kidney damage (creatinine) were increased after HS in the rats although histopathological examination of these and other tissues did not indicate visible damage. In contrast, AGS showed no indication of organ damage from either blood serum markers or histopathological examination, regardless of season. This differs from other studies where they found that after I/R, organ damage (excluding brain) occurred in the summer but not the IBA animals (15-17, 19). Several organs have been examined for resistance to I/R injury in ground squirrels, including liver, kidney, and small intestine. These organs were found to be resistant against I/R injury during the winter season, but prone in the summer season. The discrepancy may lie in the handling of the animals prior to and during the experiment as discussed above. It may also stem from a species difference. AGS are extreme hibernators whose core body temperature can reach -2.9°C during bouts of torpor (29). The plasticity

required for such extreme tolerances of body temperature may also extend to an increased ability to withstand I/R as the animal arouses from sub-freezing temperatures to normothermia. Also considering the extremely short summers in the Arctic, it may be more energy efficient to maintain the biochemical pathways needed in the winter to withstand such perturbations rather than to expend resources to tear down and reconstruct pathways solely to be utilized in the summer.

Both IBA and EU AGS had similar HS, survival

To further distinguish the ability of AGS to resist I/R injury, survival after HS was assessed, up to 72 hours. Rats were unable to maintain viability three hours past HS with three out of four animals dying within 2.5 hours and one perishing by 3.5 hours. Conversely, after HS both IBA and euthermic AGS survived with no apparent physiological deficit out to 72 hours. Although both euthermic and IBA AGS had lower initial MAP, the approximate percentage of total blood removed to achieve and maintain a MAP of 35 mm Hg was similar to that of the rats (~35% total blood volume). AGS did return to their initial MAP by the end of the experiment. Sham animals also had a low MAP (data not shown) and maintained it throughout the experiment. Previous work in our lab has determined that the unanaesthetized resting MAP of AGS is ~100 mm Hg. These data suggest that while the AGS may normally have a MAP close to that of the control rats, they are able to tolerate extended durations of low pressure without adverse effects. This may be an adaptive mechanism that the animals normally utilize during torpor bouts when HR and MAP both plummet to near zero. However, that the animals are able to tolerate this MAP when HR is not depressed is a novel finding and may contribute to their ability to tolerate I/R. Previous studies using AGS did not show a reduced MAP under anesthesia. Further studies are needed to determine the cause of the low blood pressure during anesthesia and what role it may play in resistance to I/R injury.

Independent of season, hypothermia

Overall, we found that AGS were resistant to MOF after I/R in a seasonally-independent manner. Heterothermic animals have evolved multiple adaptations to withstand the extremes

their systemic and cellular physiologies are exposed to during cooling and rewarming. Such adaptations most likely include those that impart tolerance to ischemia and reperfusion as is thought to occur during arousal from torpor. Presently, the role of seasonality in this protection and the involvement of hypothermia during ischemia is unclear. In several species I/R tolerance in isolated organ preparations has only been observed during the winter season. The animals used in previous studies were housed in varying conditions throughout the year. In the winter/hibernation season, ambient temperature was kept at $\sim 2^{\circ}\text{C}$ while during the summer/euthermic season animals were housed at 20°C . In the present study, all HS AGS were maintained at 2°C year round to remove the variable cold adaptation. This temperature also approximates burrow temperatures. In the wild, summer AGS spend less than twelve hours above ground each day (30). Most of their time is spent in subterrestrial burrows where the surrounding soil temperature is between -2°C in May and 5°C in August (31, 32). Thus, being housed at 2°C during the euthermic/summer season is a more natural environment. Being housed at 20°C may be stressful to the animals; this stress could contribute to their being less suited to tolerate I/R.

Previous studies have demonstrated that dietary restriction imparts tolerance against I/R injury (33), perhaps due to the subsequent reduction in metabolism and fall in body temperature. AGS do not eat during their interbout arousals. To reduce possible affects of the difference in food intake between the IBA AGS and the euthermic AGS and rats, all animals were fasted for 20 hours prior to surgery.

Finally the variable of hypothermia-induced protection was removed as all animals' core and temporalis muscle temperatures were maintained between 36.5 and 37.5°C for the duration of the experiments. With the variation in housing, diet, and hypothermia removed, both IBA and euthermic AGS were found to tolerate I/R injury in both clinical models. This finding is consistent with recent studies of CA induced - I/R in the AGS where euthermic AGS were found to be resistant to brain injury after CA even when maintained between 36.5 and 37.5°C during I/R.(20, 34)

Conclusion

The present study demonstrates the AGS in both the winter and summer season are resistant to organ damage, systemic inflammation, and MOF after I/R and this resistance is not dependent on their ability to cool and become hypothermic during insult. The results indicate that an altered in metabolic response during I/R may contribute to the AGS's protection. More studies are needed to examine the role of metabolism as a mechanism of I/R tolerance in the AGS. Although there are most likely numerous and redundant mechanisms against I/R injury during the winter, heterothermic season, protective mechanisms used during euthermia could provide more readily obtainable therapeutic targets for avoiding organ damage after I/R conditions in other organisms, such as humans, which are not innately tolerant.

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Tables and Figures

Table 2.1. Physiological parameters returned to pre cardiac arrest values in AGS and rats after cardiac arrest. Data shown as mean \pm S.E.M. * $p < 0.05$, Tukey, rat versus AGS.

Group	Variable	Ischemia	
		Before	After
AGS (n=4-5)	Body Weight	616 \pm 29.62	
	pH	7.47 \pm 0.01	7.47 \pm 0.05
	P _{CO2} mmHg	39.3 \pm 1.74	37.84 \pm 3.81
	P _{O2} mmHg	54.2 \pm 3.41	44.4 \pm 1.17
	HCO ₃ ⁻ mmol/L	28.48 \pm 0.65	28.53 \pm 1.05
	BE mmol/L*	5.00 \pm 0.71	4.75 \pm 1.49
	Plasma glucose mg/dL	177.4 \pm 8.34	213.4 \pm 9.20
Rat (n=5)	Body Weight	265.2 \pm 6.67	
	pH	7.44 \pm 0.01	7.47 \pm 0.02
	P _{CO2} mmHg	38.2 \pm 0.83	37.34 \pm 0.74
	P _{O2} mmHg	119.4 \pm 4.24	114.4 \pm 7.02
	HCO ₃ ⁻ mmol/L	25.68 \pm 0.57	27.02 \pm 0.88
	BE mmol/L*	1.20 \pm 0.80	3.60 \pm 1.17
	Plasma glucose mg/dL	189.4 \pm 21.10	191.25 \pm 9.28

Table 2.2. Physiological parameters change for all groups during hemorrhage and resuscitation. AGS had higher pH, BE, P_{O_2} , and HCO_3^- than rats. Plasma glucose levels were higher for AGS-IBA than rat at the end of reperfusion and three hours after reperfusion and AGS-EU were lower than rat at the start of hemorrhage (* $p < 0.05$, Tukey). ** $n=5$ for AGS-IBA P_{O_2}

Group	Variable	Hemorrhagic Shock		
		Before	End Reperfusion	3 hr After
AGS-IBA (n=6-9**)	Body Weight		691.14 ± 98.78	
	pH	7.58 ± 0.03*	7.52 ± 0.03*	7.51 ± 0.03*
	P_{CO_2} mmHg	35.87 ± 1.87	37.01 ± 2.16	35.74 ± 3.38
	P_{O_2} mmHg	53.67 ± 4.37*	50.00 ± 4.79*	54.00 ± 7.24*
	HCO_3^- mmol/L	34.32 ± 2.40*	31.46 ± 2.47*	31.03 ± 1.96*
	BE mmol/L	12.50 ± 2.81*	8.80 ± 2.87*	8.33 ± 2.28*
	Plasma glucose mg/dL	189.14 ± 9.04	229.29 ± 11.12*	221.43 ± 13.01*
AGS-EU (n=6-7)	Body Weight		662.40 ± 78.45	
	pH	7.51 ± 0.02*	7.47 ± 0.02*	7.50 ± 0.02*
	P_{CO_2} mmHg	43.00 ± 2.10	41.00 ± 2.71	36.45 ± 3.42
	P_{O_2} mmHg	54.67 ± 2.39*	50.58 ± 5.41*	45.17 ± 5.82*
	HCO_3^- mmol/L	34.60 ± 1.16*	31.60 ± 2.54*	27.35 ± 1.48*
	BE mmol/L	11.50 ± 1.20*	7.50 ± 2.64*	4.67 ± 1.20*
	Plasma glucose mg/dL	142.83 ± 10.33*	164.67 ± 13.35	164.67 ± 18.29
Rat (n=6-8)	Body Weight		379.88 ± 11.12	
	pH	7.45 ± 0.01*	7.36 ± 0.02*	7.32 ± 0.02*
	P_{CO_2} mmHg	36.72 ± 0.73	34.62 ± 1.46	35.02 ± 1.80
	P_{O_2} mmHg	112.13 ± 2.07*	122.29 ± 5.49*	126.33 ± 12.36*
	HCO_3^- mmol/L	25.27 ± 0.49*	19.62 ± 0.33*	18.60 ± 0.46*
	BE mmol/L	3.00 ± 0.00*	-5.00 ± 1.00*	-7.00 ± 0.00*
	Plasma glucose mg/dL	218.67 ± 8.18*	155.33 ± 8.89*	114.67 ± 12.16*

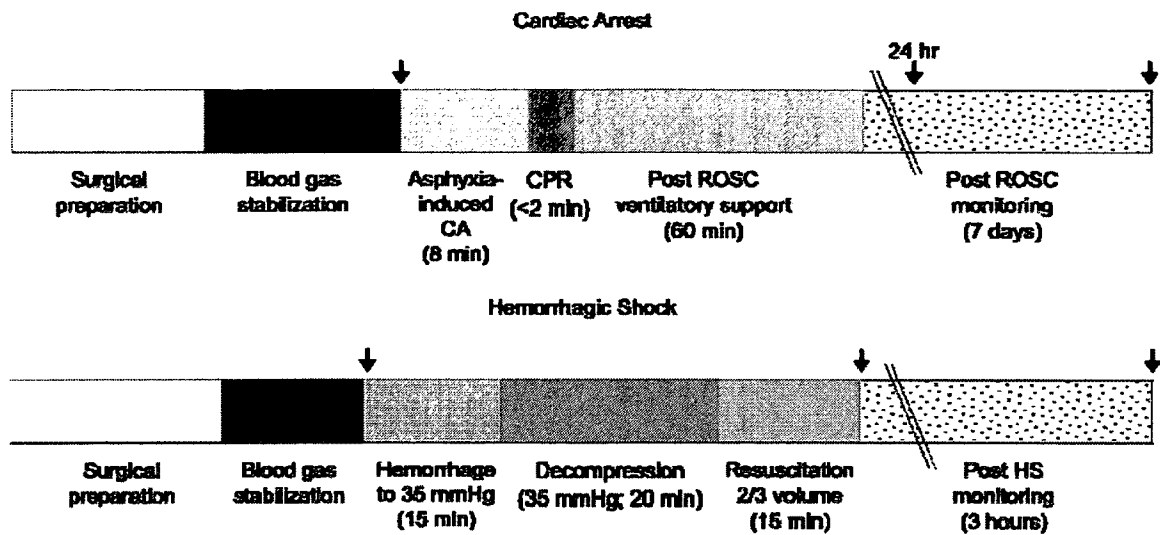


Figure 2.1. Experimental protocols for ischemia/reperfusion; CA, cardiac arrest; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation; HS, hemorrhagic shock. Core body temperature and head temperature were maintained between 35.5 and 37.5°C from the start of surgical preparation until the start of post ROSC or post HS monitoring. Arrows indicate blood sampling timepoints.

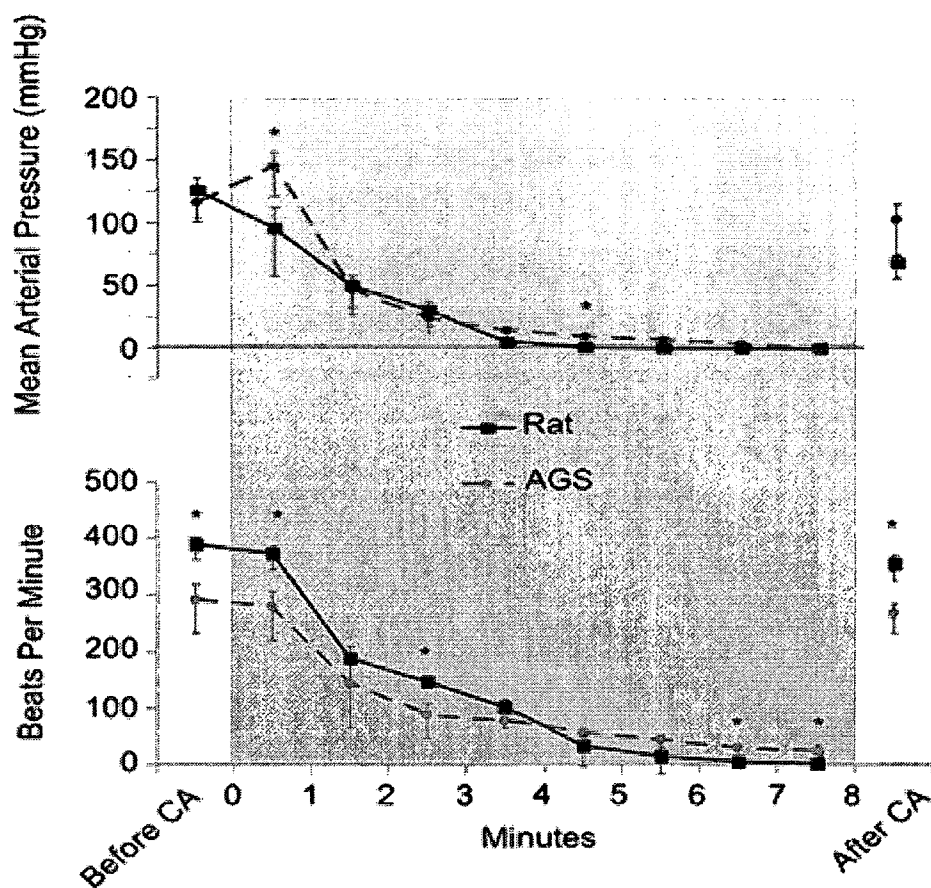


Figure 2.2. The mean arterial blood pressure (MAP; top) and average heart rate (HR; bottom) decrease similarly for both AGS and rat and return to near normal after CA. Darkened area represents period of asphyxia. MAP and HR were recorded immediately before asphyxiation (Before CA) and one hour after CA (After CA). Data shown as mean \pm S.E.M., * Tukey, $p < 0.05$ between rat and AGS. For both groups $n = 5$.

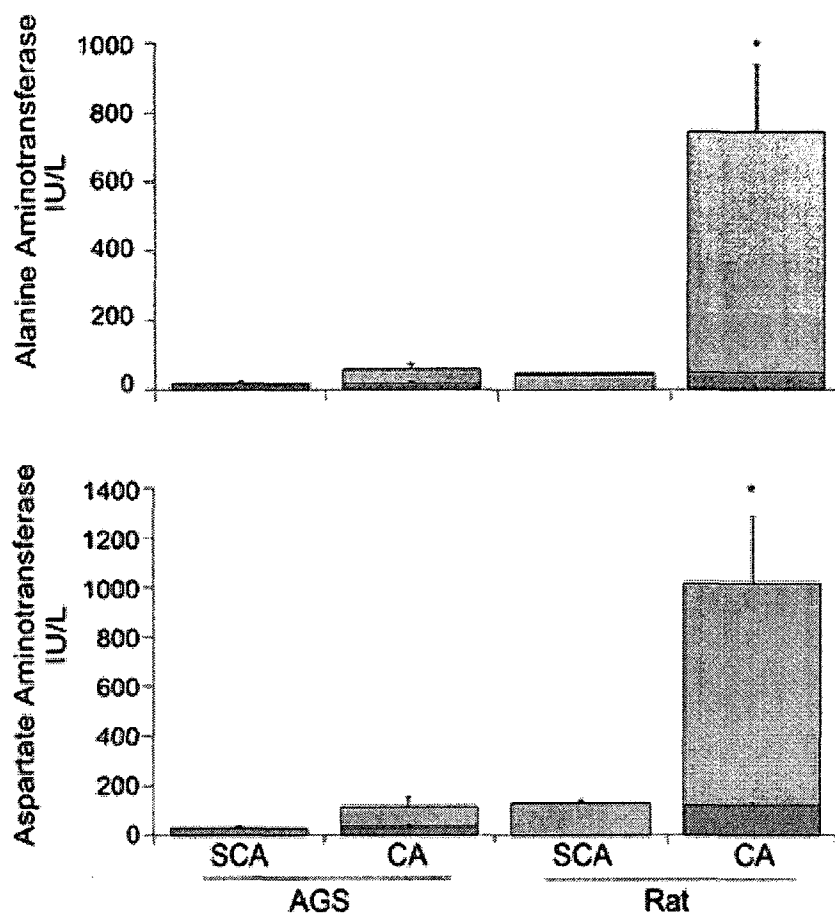


Figure 2.3. AGS did not show an increase in blood serum markers for liver damage one hour after CA. Rats had an increase in both ALT (top, * Tukey, $p < 0.05$) and AST (bottom, * Tukey, $p < 0.05$) one hour after CA compared to all other groups. Dark bars indicate baseline values. Light bars are values one hour after CA. Raw data is shown as mean \pm S.E.M. Statistical analysis was done on the difference between baseline and one hour after CA. For all groups $n=5-6$.

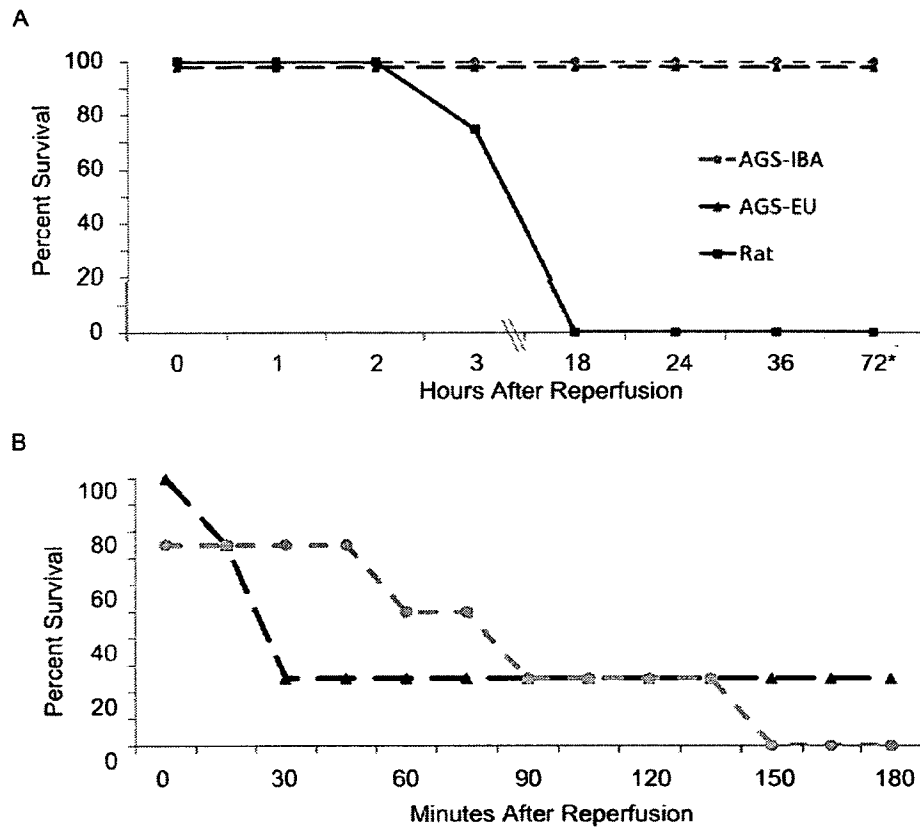


Figure 2.4. Percent survival after hemorrhagic shock. Extended survival assessed up to 72 hours after hemorrhagic shock (MAP ~35 mmHg for 20 min) in euthermic AGS, interbout arousal AGS, and rats (control; n=4 for all groups; A). Survival up to three hours after 55-60% blood loss in euthermic- (n=4) and interbout arousal (n=4) AGS (B).

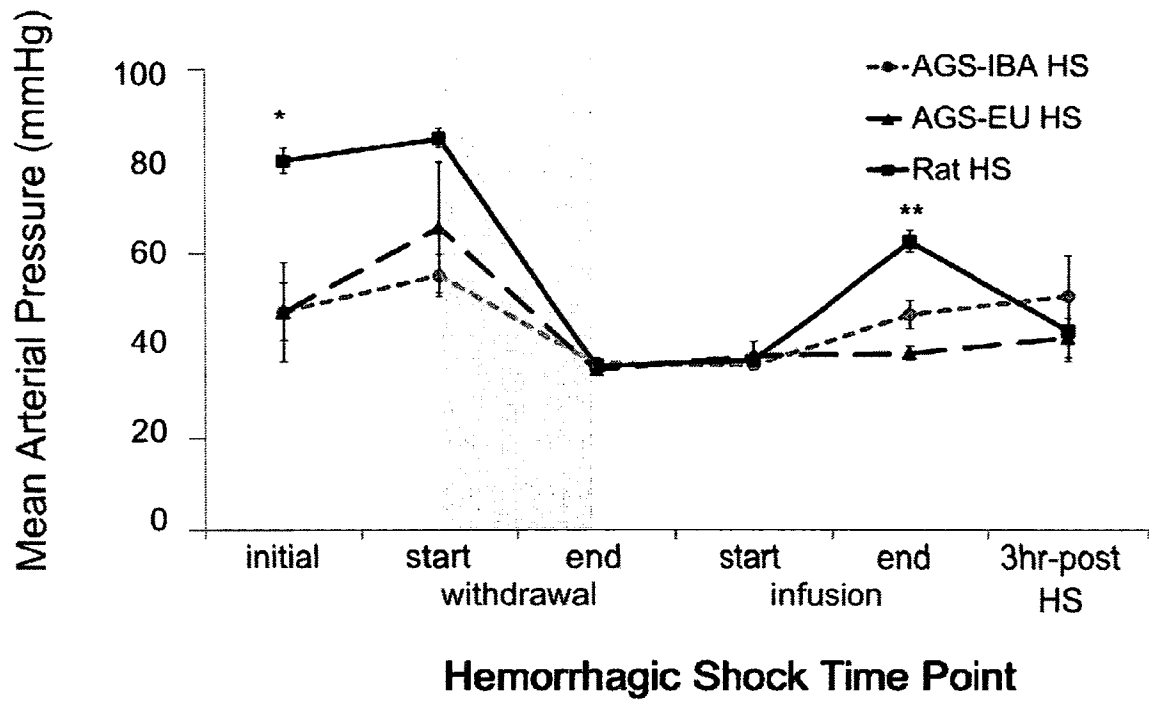


Figure 2.5. Mean arterial pressure decreases to the same mmHg for all groups during HS and is restored to varying degrees with ringers infusion. Shaded region indicates period of blood withdrawal. * Tukey, $p < 0.05$ between rats and both EU- and IBA-AGS, ** Tukey, $p < 0.05$ between all groups. Data shown as mean \pm S.E.M., $n = 6-7$ for all groups.

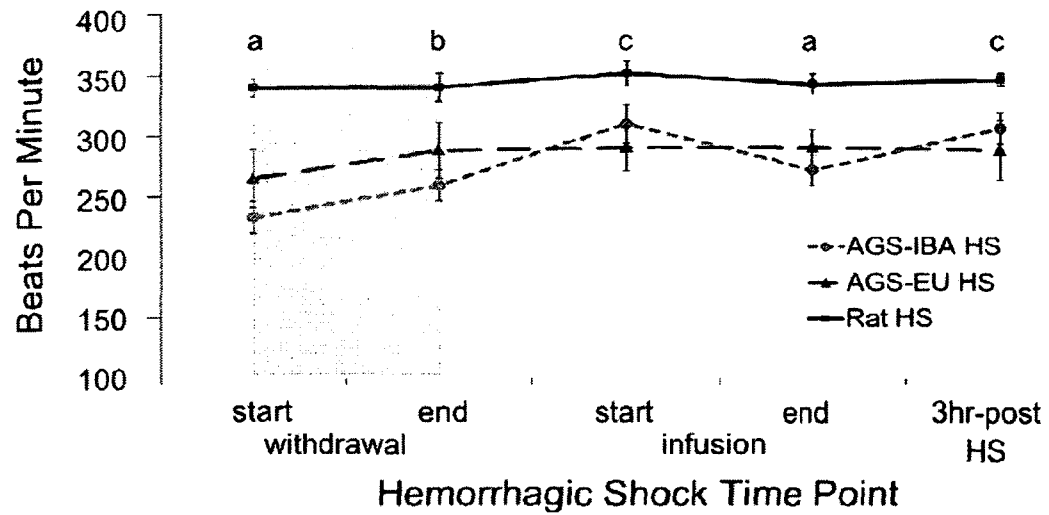


Figure 2.6. Rats have a higher average heart rate before, during and after HS than AGS. Shaded region indicates period of blood withdrawal. Rats differed from both AGS groups (a, Tukey, $p < 0.05$), just the AGS-IBA (b, Tukey, $p < 0.05$), or AGS-EU (c, Tukey $p < 0.05$). AGS-EU and -IBA did not differ from each other at any timepoint. Data shown as mean \pm S.E.M., $n = 6-7$ for all treatment groups.

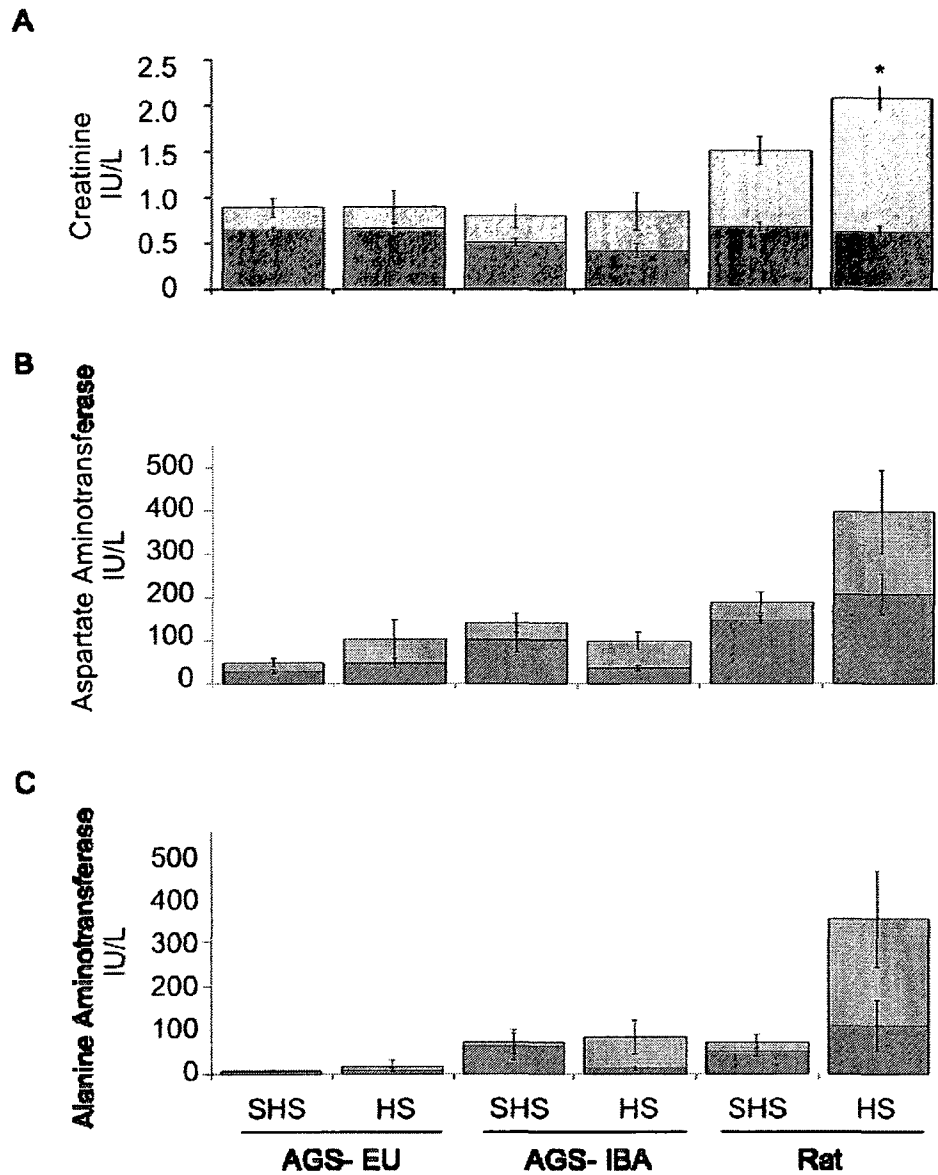


Figure 2.7. Serum markers of organ damage after hemorrhagic shock indicate that the AGS do not sustain kidney or liver damage. Dark areas indicate baseline levels obtained at the start of hemorrhage. Light areas are the levels three hours after end of resuscitation. Raw data is shown and expressed as mean \pm S.E.M. Statistical analysis with an ANOVA was performed on the difference between three hours after resuscitation and baseline. *Tukey, $p < 0.05$ between SHS and HS; $n = 4 - 8$.

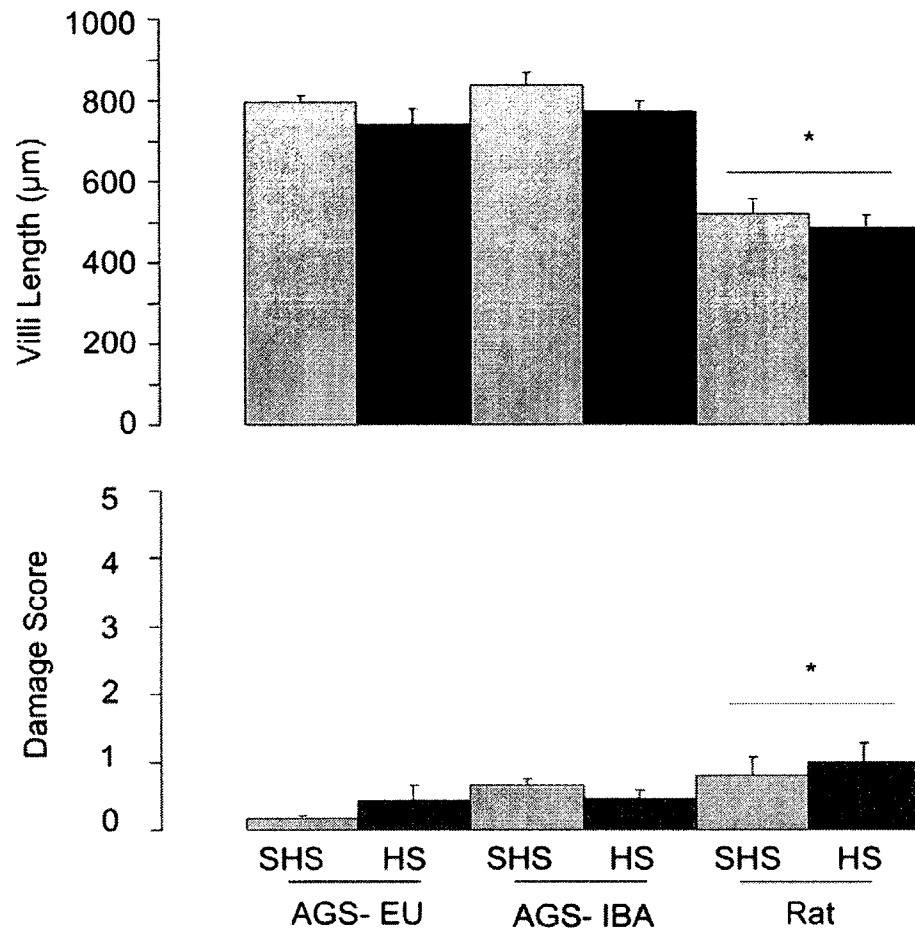


Figure 2.8. Small intestine mucosal layer did not sustain damage three hours after HS as assessed by villi length (top) and histological analysis (bottom). Data shown as mean \pm S.E.M.; * p < 0.05, Tukey Rats versus AGS-EU and AGS-IBA.

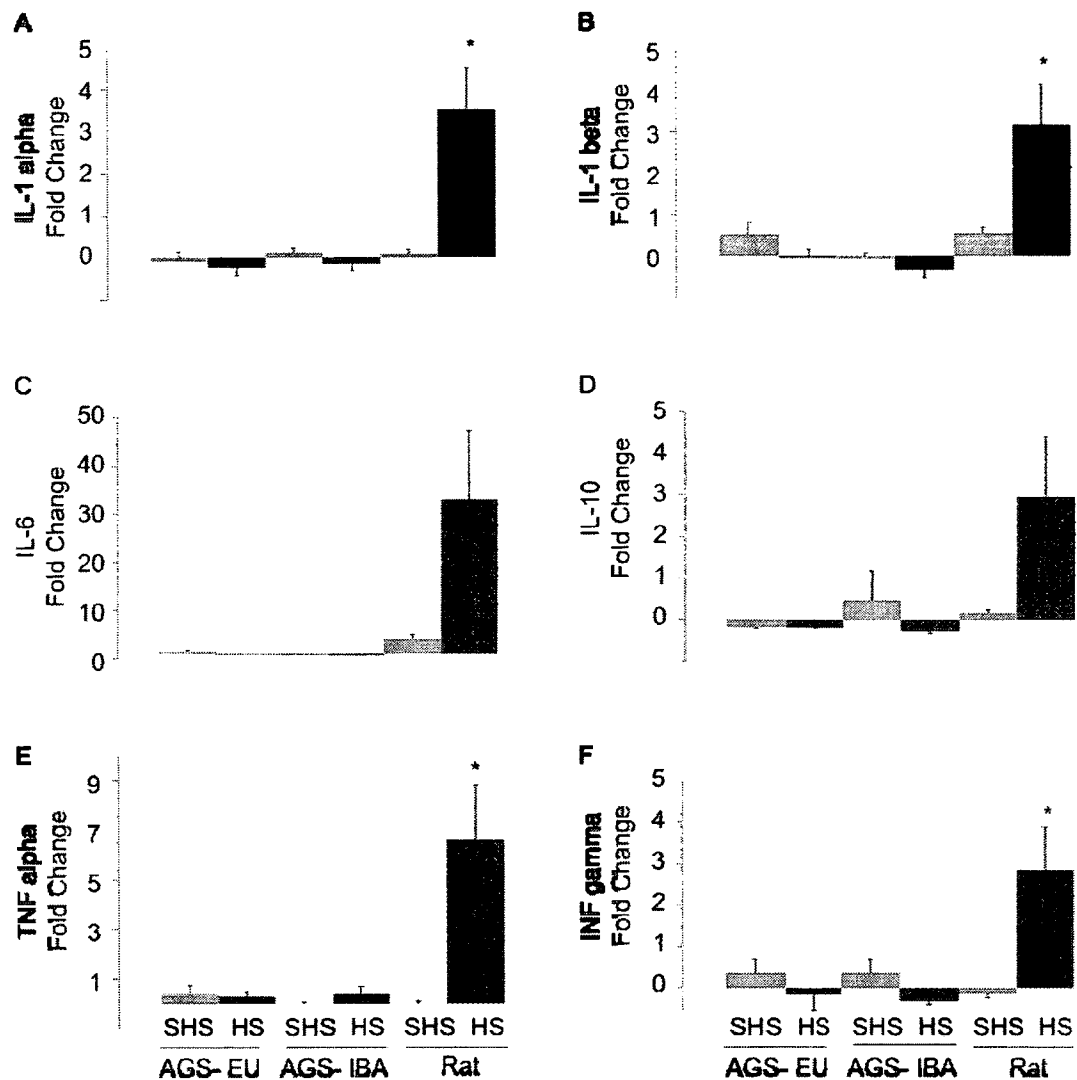


Figure 2.9. AGS do not have a significant fold change in plasma cytokine levels immediately prior to hemorrhage and three hours after resuscitation. Cytokines levels assessed for: IL-1 alpha (A), IL-1 beta (B), IL-6 (C), IL-10 (D), TNF-alpha (E), and INF-gamma (F); * $p < 0.05$, Tukey versus corresponding sham; $n = 6 - 8$ for each group.

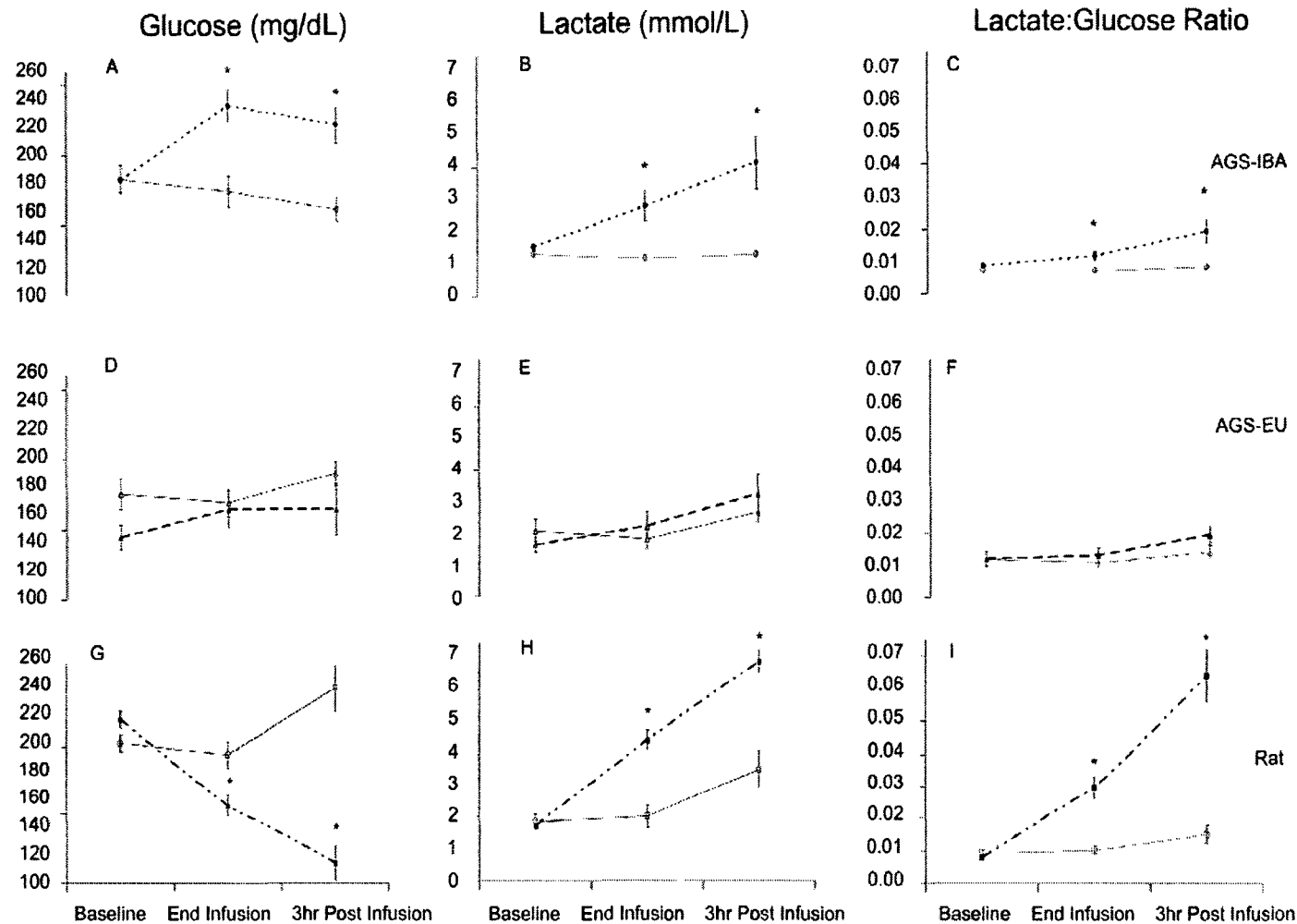


Figure 2.10. AGS maintain lower blood lactate:glucose ratio throughout HS and reperfusion. Blood glucose, lactate, and lactate:glucose ratio, before, during and after HS. Solid lines are SHS, dashed lines are HS. * Tukey, $p < 0.05$ between SHS and HS. Naïve values for glucose were 162.29 ± 10.94 , 153.88 ± 10.69 , 205.67 ± 7.78 mg/dL; lactate 1.66 ± 0.29 , 1.48 ± 0.25 , 1.92 ± 0.20 mmol/L; for AGS-EU, AGS, IBA, and rats, respectively.

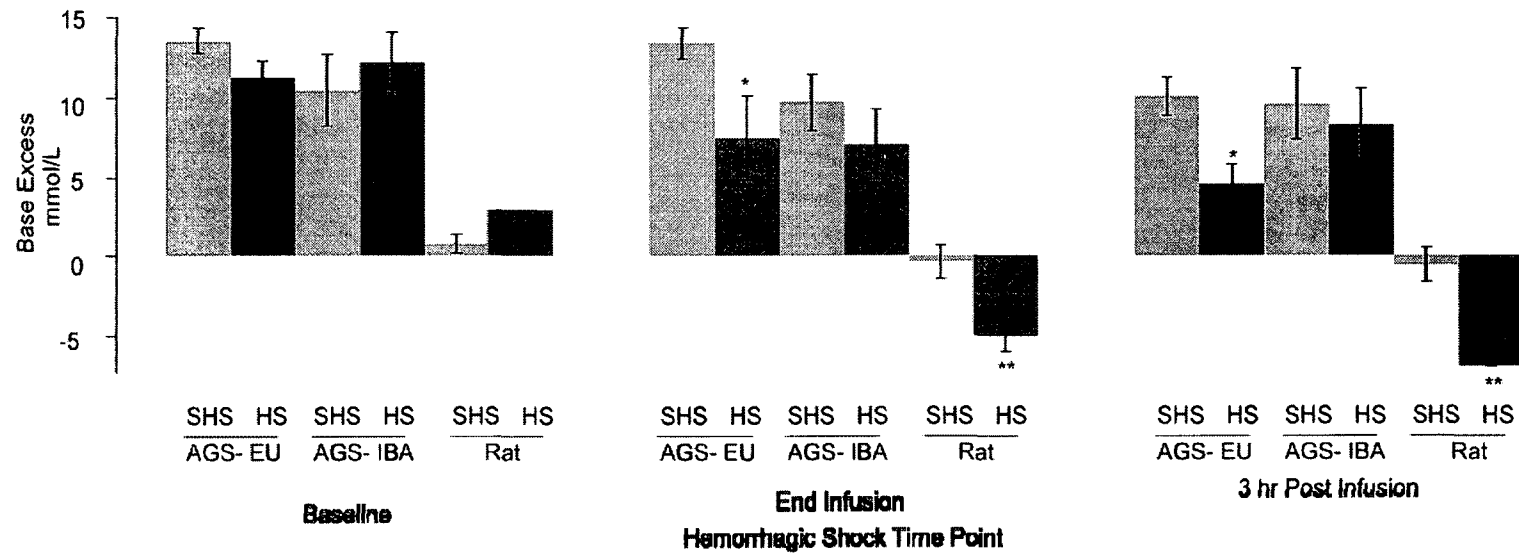
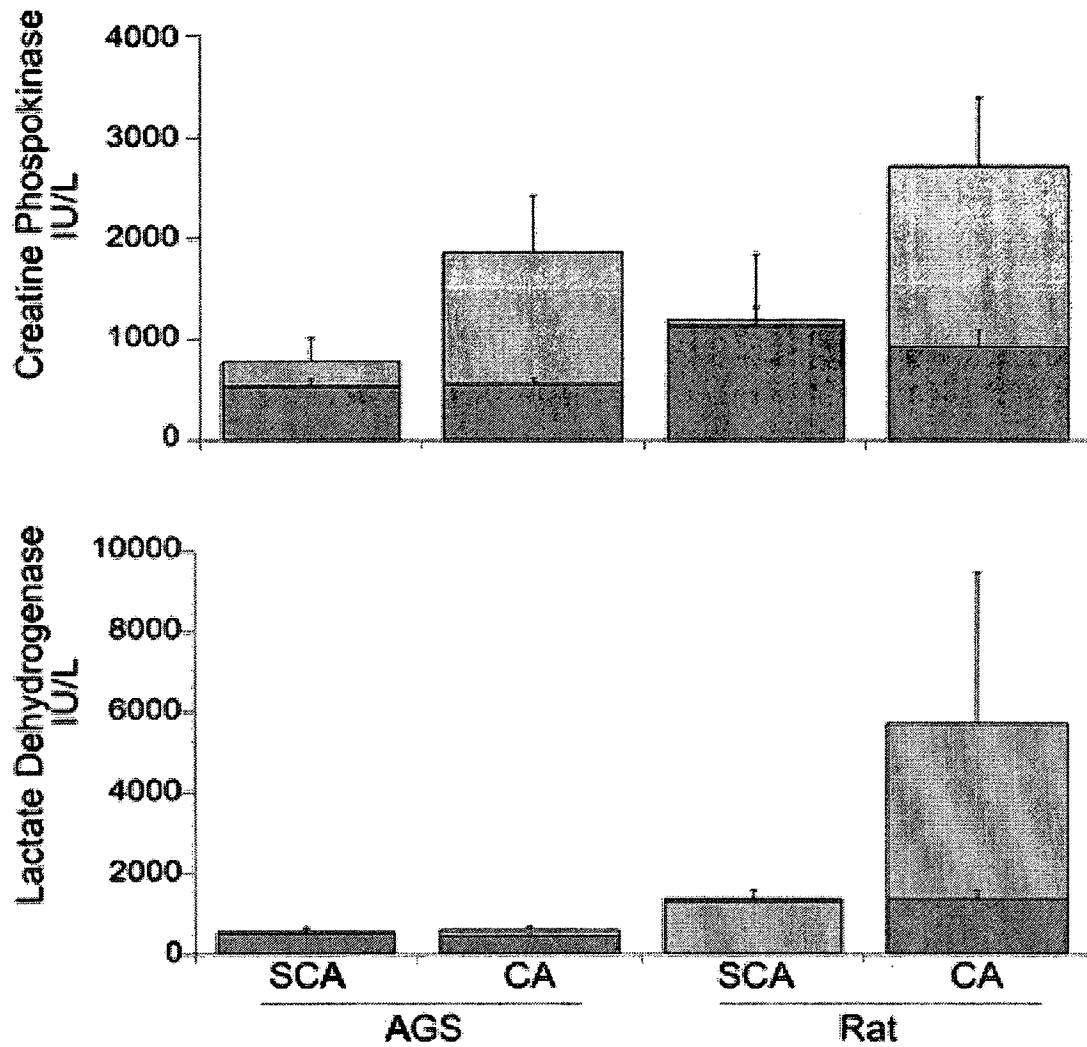
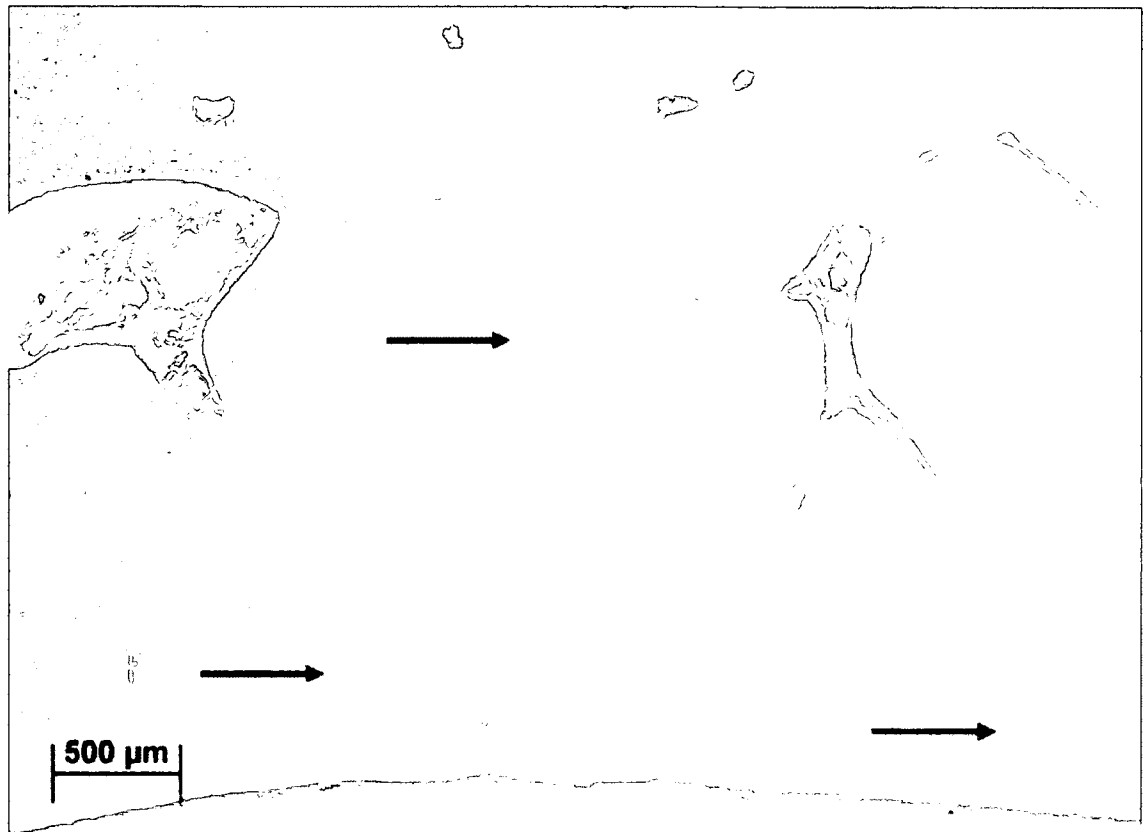


Figure 2.11. AGS maintain positive whole blood base excess values before, during, and after HS. Data presented as mean±S.E.M.; * Tukey, $p<0.05$ between AGS-EU Sham HS (SHS) and HS, ** Tukey, $p<0.05$ between rat SHS and HS.



Supplemental Figure 2.1. Changes in circulating CPK and LDH levels before and 24 hours after CA. Dark bars indicate baseline values. Light bars are values after CA. Raw data is shown as mean \pm S.E.M.. $n=5-6$ for all groups except AGS SCA CPK $n=2$ and rat CA LDH $n=3$.



Supplemental Figure 2.2. Multiple foci of ischemic necrosis in rat liver after CA. Arrows indicate areas of necrotic cells, 4x magnification.

Supplemental Table 2.1. Characteristics of AGS undergoing CA during the summer (euthermic) season.

Animal number	09-03	09-04	09-47	09-35	09-84	09-10	09-67
Experimental Group	CA						
Age	Adult	Adult	Adult	Adult	Adult	Adult	Adult
Sex	Male	Male	Female	Female	Male	Male	Male
Mass (g)	724	601	491	569	563	564	628
Experiment day	26-May-10	27-May-10	6-Jun-10	7-Jun-10	11-Jun-10	14-Jun-10	28-Jun-10
Last day of torpor during previous season	7-Apr-10	no hib	26-Mar-10	13-Mar-10	14-Mar-10	19-Feb-10	no hib

Supplemental Table 2.2. Characteristics of AGS undergoing HS during the winter (IBA) season.

Animal number	09-56	08-83	08-40	09-07	08-68	10-02	10-08	10-05	10-48
Season	Winter								
Age	Adult	Adult	Adult	Adult	Adult	Adult	Adult	Adult	Adult
Sex	Female	Female	Female	Male	Male	Female	Female	Female	Female
Mass (g)	862	1123	1044	556	1056	461	441	559	721
First day of spontaneous torpor	18-Aug-10	18-Aug-10	17-Aug-10	19-Aug-10	4-Aug-10	25-Aug-10	22-Aug-10	29-Aug-10	2-Oct-10
Experiment day	7-Jan-11	11-Jan-11	24-Jan-11	25-Jan-11	27-Jan-11	1-Feb-11	10-Feb-11	17-Feb-11	18-Feb-11
No. of spontaneous torpor bouts prior to HS	11	16	14	11	14	14	10	13	13
Ave length of previous 3 torpor bouts (days)	14.67	10.67	11.33	8.33	13.67	12.33	20.33	17.67	17.67
Day in bout	6	4	4	8	4	5	13	2	11
Tb at induced arousal (°C)	3	3.3	2.9	3.3	3	3.3	3.5	4	4.7
Blood volume removed (% total)	16	15	22	56	24	58	42	28	37

Supplemental Table 2.3. Characteristics of AGS undergoing HS during the summer (euthermic) season.

Animal number	10-26	10-46	10-06	10-44	09-59	09-54
Season	Summer					
Age	Adult	Adult	Adult	Adult	Adult	Adult
Sex	Male	Male	Female	Male	Female	Female
Mass (g)	856	549	580	619	591	673
Last day of torpor during previous season	26-Jan-11	10-Feb-11	24-Feb-11	23-Feb-11	12-Mar-11	5-Apr-11
Experiment day	9-Jun-11	14-Jun-11	23-Jun-11	24-Jun-11	8-Jul-11	14-Jul-11
Blood volume removed (% total)	22	28	62	40	34	38

Supplemental Table 2.4. Characteristics of AGS undergoing SHS during the winter (IBA) season.

Animal number	08-51	09-86	08-97	10-09	10-24	10-28	10-33	10-12
Season	Winter							
Age	Adult	Adult	Adult	Adult	Adult	Adult	Adult	Adult
Sex	Female	Male	Female	Female	Male	Male	Male	Female
Mass (g)	671	704	904	454	738	729	731	438
First day of spontaneous torpor	7-Aug-10	18-Aug-10	17-Aug-10	29-Aug-10	7-Dec-10	16-Oct-10	1-Nov-10	16-Sep-10
Experiment day	21-Jan-11	18-Jan-11	19-Jan-11	28-Jan-11	3-Feb-11	4-Feb-11	11-Feb-11	23-Feb-11
No. of spontaneous torpor bouts prior to SHS	12	13	11	12	5	10	8	11
Ave length of previous 3 torpor bouts (days)	9.67	14	17.67	14.33	13.67	17.33	13.67	14
Day in bout	3	5	13	8	7	6	7	5
Tb at induced arousal (°C)	3.1	not taken	3.3	4	3.7	4.2	3.8	3.7

Supplemental Table 2.5. Characteristics of AGS undergoing SHS during the summer (euthermic) season.

Animal number	09-76	09-83	10-16	10-27	10-14	09-68	10-30
Season	Summer						
Age	Adult	Adult	Adult	Adult	Adult	Adult	Adult
Sex	Female	Male	Male	Male	Female	Female	Female
Mass (g)	1036	554	464	658	617	833	446
Last day of torpor during previous season	10-Nov-10	10-Feb-11	20-Feb-11	09-Feb-11	28-Feb-11	5-Jan-11	27-Feb-11
Experiment day	7-Jun-11	10-Jun-11	16-Jun-11	21-Jun-11	28-Jun-11	29-Jun-11	15-Jul-11

Supplemental Table 2.6. Histopathology organ damage scores for HS experiments. No significant difference in organ damage found due to HS treatment. Data given as mean±S.E.M., n=6-8 for all groups.

	AGS-EU			AGS-IBA			Rat		
	Naïve	SHS	HS	Naïve	SHS	HS	Naïve	SHS	HS
Intestine	0.39±0.26	0.93±0.42	0.08±0.08	0.04±0.03	0.19±0.19	0.03±0.03	0.84±0.22	2.31±0.53	1.94±0.39
Lung	1.18±0.44	3.04±1.29	2.88±0.90	0.94±0.25	1.22±0.45	2.19±0.60	1.09±0.42	0.94±0.34	1.66±0.54
Kidney	4.75±2.02	4.07±1.00	3.79±0.84	1.16±0.63	2.13±0.68	3.19±1.19	8.25±1.12	9.31±20.5	11.97±2.74
Spleen	3.11±1.15	2.86±0.47	3.75±0.85	3.59±0.45	3.25±0.70	3.12±1.10	11.44±1.59	11.75±1.10	11.81±1.31
Liver	3.54±0.96	5.21±1.66	6.54±1.61	4.50±0.70	5.69±1.88	5.03±0.56	3.03±1.21	5.06±0.79	5.94±1.09

Supplemental Table 2.7. Cytokine concentration values (pg/mL) prior to hemorrhage and three hours after hemorrhage.

	AGS-EU				AGS-IBA				Rat			
	SHS		HS		SHS		HS		SHS		HS	
	Prior	After	Prior	After	Prior	After	Prior	After	Prior	After	Prior	After
IL-1 alpha	72.81	58.93	102.51	74.29	56.48	60.97	53.46	43.45	21.82	23.10	20.17	92.91
IL-1 beta	15.26	20.01	5.25	6.44	15.20	16.35	15.10	10.51	30.54	63.20	29.78	113.76
IL-6	16.83	20.53	13.85	10.95	16.65	16.15	18.81	17.91	35.07	139.09	31.11	1170.82
IL-10	68.02	54.81	61.93	49.86	60.60	54.56	67.20	46.43	108.87	121.00	96.66	404.17
TNF-alpha	2.72	3.15	1.85	2.5	4.26	3.33	3.04	3.27	5.82	5.58	6.10	46.52
INF- gamma	3.07	3.98	7.67	3.05	9.04	3.77	12.91	6.52	4.29	3.53	3.55	12.78

Chapter 3. ¹H-NMR Metabolomic Biomarkers of Poor Outcome after Hemorrhagic Shock are absent in Hibernators³

Abstract

Objective: Hemorrhagic shock following trauma is a leading cause of death among persons under the age of 40. During hemorrhagic shock, the body undergoes systemic ischemia followed by reperfusion during medical intervention. Ischemia/reperfusion (I/R) results in a disruption of cellular metabolic processes that ultimately lead to tissue and organ dysfunction or failure. Resistance to I/R injury is a characteristic of hibernating mammals. The present study sought to determine if arctic ground squirrels (AGS) are protected from hemorrhagic shock (HS)-induced global I/R injury due to the ability to maintain a stable metabolic profile during HS.

Design: Non-randomized controlled trial.

Setting: Laboratory.

Subjects: Sprague-Dawley rats, euthermic AGS, and interbout arousal AGS.

Interventions: Organism's innate resistance to I/R injury.

Measurements and Main Results: Rats and AGS were subject to hemorrhagic shock by withdrawing blood to a MAP of 35 mm Hg and maintaining the low MAP for 20 min before reperfusing with Ringers. The animals' temperature was maintained at 36.5-37.5°C for the duration of the experiment. Plasma samples were taken immediately before hemorrhage and three hours after reperfusion. Total plasma and lipid fractions from plasma were then analyzed via ¹H-NMR for hydrophilic and lipophilic metabolites, respectively. Rats, animals susceptible to I/R injury, had a qualitative shift in their hydrophilic metabolic fingerprint and had alterations in

³ Bogren LK, Murphy CJ, Johnston EL, Drew KL, prepared for submission to Critical Care Medicine, 2013.

several metabolites during I/R indicative of metabolic adjustments and organ damage. In contrast, I/R injury resistant AGS, regardless of season, were able to maintain a qualitative ^1H -NMR metabolic profile with little (winter/interbout arousal AGS) or no (summer/euthermic) changes in quantified metabolites during HS-induced global I/R.

Conclusions: AGS, regardless of hibernation season, are resistant to disruptions in their metabolic processes and organ damage during HS- I/R, independent of hypothermia.

Introduction

Hemorrhagic shock (HS) is a serious medical problem most often caused by trauma. Trauma is the third leading cause of death for persons under the age of 40 with approximately one third of these deaths resulting from HS (1, 2). During HS, the massive loss of blood reduces systemic blood pressure and produces ischemia/reperfusion (I/R) injury. Hypoperfusion of the tissues leads to an inadequate supply of oxygen and nutrients in addition to the inability to remove waste products resulting in cellular dysfunction. This is then compounded with the generation of reactive oxygen species during reperfusion that leads to further tissue damage (3, 4).

Hibernating animals, such as ground squirrels, have been shown to have an innate resistance to I/R injury. This resistance is found not only in isolated organ I/R, but also in systemic I/R as is found in HS (5-11). The arctic ground squirrel (*Urocitellus parryi*, AGS) experience HS-induced I/R without tissue damage or multi organ failure, independent of their hibernation season or ability to become hypothermic (5). There is also a well-documented ability in ground squirrels to switch fuel sources from carbohydrate- to lipid-based metabolism during their hibernation season (7, 12-22). Previous studies in our lab indicated that, in conjunction to their resistance to HS-I/R injury, arctic ground squirrels (AGS) also have an altered glucose/lactate and base excess response during HS as compared to I/R injury-prone species,

indicating that they may have a different metabolic response to I/R (5). Differences in metabolic process during I/R in the injury resistant species versus an injury prone species is not known and may provide valuable information as to the molecular basis of the protective phenotype.

The purpose of this study was to identify changes in circulating metabolic profiles and metabolites that would be indicative of metabolic pathways in the hibernators that might contribute to their innate protective mechanism against I/R injury and multi organ failure. We hypothesized that we would be able to identify several circulating metabolites in the rat that could serve as biomarkers for metabolic alterations leading to poor outcome after HS and that these biomarkers would be absent in the AGS. Specifically, we expected to see differential activation of glucose and anaerobic metabolism in the I/R injury prone rat and not in the I/R-injury resistant AGS.

¹H-NMR-metabolomic analysis of total plasma samples and their hydrophobic fractions taken before and after HS-I/R was conducted for summer/euthermic (EU) and winter/interbout arousal (IBA) AGS and compared to I/R injury prone rats. Spectral patterns were qualitatively compared for overall changes in the metabolic “fingerprints” for each group followed by quantification of specific metabolites identified in the spectra. We found that during HS-I/R, both EU- and IBA- AGS are able to maintain their hydrophilic and hydrophobic metabolomic ¹H-NMR-spectral fingerprints and resist I/R-induced changes to metabolites involved in carbohydrate turnover, anaerobic metabolism, and organ dysfunction.

Methods

The qualitative metabolic profiles and concentrations of specific metabolites were evaluated from naïve, sham, and hemorrhaged animals. These parameters from each experimental condition were then compared between summer/euthermic, winter/IBA AGS, and the I/R injury-prone control species, rats.

Animals

Juvenile AGS were live trapped north of the Brooks Range (69° latitude) in Alaska during July and were maintained in captivity at least 10 months prior to the experiments. The animals were housed at 17°C under light conditions based on 69° latitude from time of capture until late August when they were then transferred to a cold chamber. Animals remained in the cold chamber (2°C) with 4:20 light:dark conditions until the experiments which were conducted between May and June (summer euthermic) and December- February (winter, interbout arousal) the following two years. Food and water were provided ad libitum but food was removed twenty hours prior to surgery. Both male and female AGS (438-1123g) were used in this study due to the availability of wild caught animals. Animals were not used during the 4-week period following the end of spontaneous torpor bouts when reproductive hormone levels are elevated (23, 24). Summer/euthermic status of an animal was assessed by body temperature, activity and lack of spontaneous torpor for at least 4 weeks. Animals were considered to be in the winter/hibernation season when they had been having regular spontaneous torpor bouts for at least eight weeks prior to the experiments (Supplemental Tables 3.1-4). I/R sensitive controls, Male Sprague-Dawley rats (350-420g), were obtained from University of Alaska Fairbanks Animal Resource Center (Sprague-Dawley, Simonson Laboratory, Gilroy, CA derived rats). All animal procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the Animal Use and Care Committee of the University of Alaska Fairbanks.

Hemorrhagic Shock

Anesthesia was induced and maintained with isoflurane and a 30:70 mixture of O₂ and N₂O. Once unresponsive, the animals were intubated with an endotracheal catheter and connected to a ventilator humidified with mucomist (1.4mL/70mL H₂O). Vital signs were monitored by EKG and a rectal (core body temp) and temporalis (head/ brain temperature) thermocouples. The animals were then cannulated in their right femoral artery and vein and left femoral artery. Mean arterial blood pressure (MAP) was measured via the femoral arterial

catheter connected to a precalibrated pressure transducer and was recorded continuously. Blood was sampled every 10 min for P_{CO_2} and P_{O_2} . Mechanically ventilated stroke volume, breaths per minute, and percent O_2 were changed until blood gases were within predetermined normal ranges as previously described (10, 11). Rectal and temporalis temperatures were monitored and heat provided by two heating lamps and a heated water pad until both temperatures were between 36.5 and 37.5°C. Blood was then withdrawn over a 15 minute period to achieve a target MAP of 35 mm Hg. The MAP was held at ~35 mm Hg for 20 minutes before animals were reperfused intravenously with non-lactated Ringers solution (148 mM NaCl, 2 mM $CaCl_2$, 4 mM KCl) equal to 2/3 volume of the blood removed during hemorrhage (Hospira, Inc., Lake Forest, IL). Animals were monitored for an additional three hours. Sham hemorrhagic shock (SHS) animals were subject to the hemorrhagic shock procedure, but blood was not removed nor Ringers administered.

EDTA-treated plasma samples were collected from the femoral artery immediately prior to HS (baseline), and by cardiac puncture at the end of the three hour monitoring period. Blood from naïve animals was collected via cardiac puncture. Samples were kept on ice and then snap frozen in liquid nitrogen before being stored at -80°C.

Proton quantitative 1H -NMR on EDTA-plasma and lipid extracts

For NMR analysis, samples of EDTA-plasma in D_2O (99.8% Alfa Aesar, Ward Hill, MA; 150:450 μ L) were transferred into 5-mm NMR tubes (Wilmad Lab Glass, Buena, NJ). 1H -NMR spectra were acquired at 5° C (based on Methanol calibration) with a 600-MHz Bruker Avance-III system running TopSpin 3.0 software (Bruker Biospin, Fremont, CA) using a dual resonance high resolution SmartProbe single axis Z-gradient. The water signal was suppressed using NOESY presaturation followed by CPMG relaxation editing ("PROF_CPMG" setting in TopSpin 3.0). A standard, trimethylsilyl propionic-2,2,3,3-tetradeuteriopropionic acid (TMSP, 5 mM in D_2O) contained in a coaxial insert and placed in the NMR tube was used for metabolite quantification of fully relaxed 1H -NMR spectra and as a 1H chemical shift reference (0.0 ppm). The 1H -NMR

peaks for single metabolites were identified and referred to published chemical shift or a metabolite chemical shift library (25-28). Two dimensional experiments including COSY, JRes, and HMQC were used to confirm assignments. After Fourier transformation, phasing, and baseline correction in TopSpin, each ^1H peak was integrated with Amix (Bruker Biospin). The absolute concentration of each metabolite was then referred to the TMSP integral and calculated according to the equation adapted from Serkova et al (25): $C_x = (I_x \cdot N_x \cdot C) / 9$. Where C_x is metabolite concentration ($\mu\text{mol/mL}$), I_x is integral of metabolite ^1H peak, N_x is number of protons in metabolite ^1H peak, C is TMSP concentration, I is integral of TMSP ^1H peak at 0 ppm (this is nine as TMSP contains nine protons). An additional correction factor of 8.68 was applied to adjust for the differences in diameters between the NMR tube and the insert (derived as the ratio of the cross-sectional areas of the tubes). The final metabolite concentrations were expressed as mM of EDTA-plasma volume.

Lipids were extracted from the EDTA-plasma samples using a dual chloroform/methanol extraction as previously described (5). Briefly, plasma was mixed 1:2 (vol/vol) with cold chloroform/methanol (1:2 vol/vol) and centrifuged. Liquid was removed from the pellet and the pellet was washed again in chloroform/methanol and centrifuged. The second supernatant was removed and combined with the first which together were washed with cold water. The mixture was centrifuged to separate the hydrophobic and hydrophilic fractions. Lipid extracts were lyophilized and reconstituted in 0.6 mL deuterated chloroform/methanol (1:2, vol/vol) for NMR analysis. TSP was contained in the deuterated methanol (0.3% 1.47 mM) and a coaxial insert was not used.

Statistics

Multivariate analysis (partial least squares-discriminate analysis; PLS-DA) was used for comprehensive metabolite profile analysis within complex ^1H -NMR spectra. PLS-DA was performed using MetaboAnalyst, a freely available, web-based application designed to support quantitative metabolomics (29, 30). Before being analyzed, the spectra were segmented into

"buckets" each with a 0.01 ppm width. The water region (4.8-5.2ppm) and EDTA regions (2.53-2.58, 3.1-3.3, and 3.6-3.7 ppm) were excluded from the bucketing and multivariate analysis of the unextracted plasma samples while the chloroform (8.0-7.5 ppm) and methanol (5.22-4.76 & 3.42-3.25 ppm) regions were excluded from the lipid spectra. Before PLS-DA, data was normalized to a Gaussian distribution (a feature in MetaboAnalyst).

All data are presented as mean \pm S.E.M.. Fold change was calculated as (final concentration - initial concentration)/ initial concentration. Absolute individual concentrations of each metabolite were analyzed via three-way ANOVA with repeated measures (SAS; Cary, NC). Metabolites with a significant time x group x treatment interaction (group defined as EU-AGS, IBA-AGS, or rat, and treatment consisting of hemorrhagic shock or sham) were further analyzed via two-way ANOVA with repeated measures. All significant effects in ANOVAs were followed by a Tukey's post-hoc test. Statistical significance was considered to be a p value of <0.05 .

Results

To test the hypothesis that AGS are resistant to the disruption in metabolic pathways associated the development of multi organ failure after global I/R, ^1H -NMR analysis of the circulating metabolites in I/R-injury prone rats and I/R-injury resistant AGS was conducted.

Metabolic ^1H -NMR spectral pattern difference between test groups

The global metabolic profile of circulating metabolites present in the plasma of naïve animals was assessed via ^1H -NMR spectral pattern comparison each of the test groups (rats, EU-AGS, and IBA-AGS) through the multivariate statistical analysis, PLS-DA. PLS-DA is a supervised classification method that maximizes the separation of groups of data based on variables in the data that are ranked on how well they explain differences in the groups. When analyzed for spectral pattern similarities using PLS-DA, each naïve group had distinct clustering (Fig. 3.1A). The clusters of the EU-AGS and IBA-AGS were closer in proximity to each other than

with the rats, indicating greater spectral similarity between the two seasonalities of AGS than either had with the control species. Most of the animals in the AGS groups clustered together; however, in each group there was a distinct outlier. The naïve rats showed less of this clustering and were more spread out. PLS-DA analysis of the HS and sham animals' spectra immediately before treatment also showed distinct clustering of each group (data not shown). When ^1H -NMR spectra of plasma samples before and after hemorrhagic shock was analyzed via PLS-DA, both EU- and IBA-AGS, before and after hemorrhagic shock overlapped one another without any distinct, segregated clusters (Fig. 3.1B). In contrast, the rats from both time points were separate from the majority of the AGS and formed two distinct groupings, one for the before HS samples and the other for the samples collected three hours after HS. This indicates that the rats experienced distinct alterations in the ^1H -NMR spectra as a consequence of HS while AGS did not.

We next looked at the ^1H -NMR spectra of the non-polar molecules. When obtaining spectra of total-plasma, a CPMG relaxation editing sequence was used to suppress the macromolecule signals (including lipids) from obscuring the hydrophilic metabolite peaks. To evaluate the possible contribution of lipid metabolism in AGS resistance to I/R-induced multi organ failure, plasma samples were lipid extracted and PLS-DA was further employed to determine spectral pattern similarities in the non-polar fraction. PLS-DA analysis of the lipophilic components of the plasma samples was conducted on naïve animals of each test group (Fig. 3.2A) and from samples taken before and three hours after HS (Fig. 3.2B). For the naïve animals, the lipid spectra for both the EU- and IBA-AGS were clustered together with the exception of an outlier in each group. The AGS clustering was distinct from the rats, which had a more scattered distribution. Lipid ^1H -NMR spectra was also evaluated before and after HS (Fig. 3.2B). Unlike the naïve animals, those subject to surgical conditions and hemorrhagic shock lacked any encapsulated clustering with the rats not forming a distinct grouping but overlapping with the EU-AGS. The EU-AGS grouping crossed into both the rat and IBA-AGS clusters, but the

rat and IBA-AGS did not intersect. Within each test group, the spectra before and after HS did not segregate, indicating there was not a spectral pattern difference before and after HS in any of the three groups.

Changes in circulating metabolite concentrations after hemorrhagic shock

Quantitative analysis of the metabolites identified in the ^1H -NMR spectra was employed to obtain more specific circulating metabolic profiles of the rats, EU-AGS, and IBA-AGS and to determine any changes in the concentration during I/R. Analysis was conducted on the untreated plasma samples (Fig. 3.3) and on the lipophilic components of the lipid-extracted samples (Fig. 3.4). Figures 3.3 and 3.4 showed representative spectra from naïve animals of each of the groups with peaks used to quantify specific metabolites identified.

A combined total of 25 metabolites were identified in the untreated and lipophilic spectra with 20 being found in the whole-plasma spectra and five being identified in the lipophilic spectra. Of the 25 metabolites, only eight had a significant change in concentration from before HS to three hours after HS that was different between group (rat, EU-AGS, or IBA-AGS) and was not found in the sham experiments ($p < 0.05$, ANOVA, time x group x treatment; Table 3.1; naïve data; Supplemental Table 3.5).

Fourteen of these 25 metabolites had a significant concentration change between the start of HS and three hours post-HS. All of these showed a significant difference between rat, EU-AGS, and IBA-AGS ($p < 0.05$, ANOVA, time x group and time x group x treatment; Table 3.1). Of these, only eight show a difference that was due to the HS ($p < 0.05$, ANOVA, time x group x treatment; Table 3.1). These included acetate, alanine, creatinine, histidine, β -hydroxybutyrate, lactate, oxaloacetate, and tyrosine. The fold change in these metabolites is shown in Figure 3.5. The I/R injury prone rats had greater increases as compared to shams in acetate ($p=0.0069$, 2-way ANOVA, time x treatment), creatinine ($p=0.0123$, 2-way ANOVA, time x treatment), lactate ($p<0.0001$, 2-way ANOVA, time x treatment), oxaloacetate ($p=0.0069$, 2-way ANOVA, time x

treatment), tyrosine ($p=0.0004$, 2-way ANOVA, time x treatment), alanine ($p=0.0002$, 2-way ANOVA, time x treatment) and less of an increase than sham in histidine ($p=0.0345$, 2-way ANOVA, time x treatment). The greatest difference was seen in lactate which had a 3.6-fold increase after HS. In contrast, EU-AGS showed no significant changes in these metabolites and IBA-AGS, showed increases only in tyrosine ($p=0.0007$, 2-way ANOVA, time x treatment) and alanine ($p=0.0245$, 2-way ANOVA, time x treatment). Interestingly, although β -hydroxybutyrate had a significant three-way ANOVA interaction ($p=0.0296$, time x group x treatment), no significant effect of group x time was found in the subsequent 2-way ANOVA (i.e. change was noted between the sham and HS animals in any group).

Discussion

In this study, plasma samples from before and after HS-induced systemic I/R were analyzed both qualitatively and quantitatively to establish biomarkers for changes in metabolic processes and organ dysfunction and injury during HS. These were then used to identify differences between I/R injury prone species and hibernators, which have been shown to be resistant to I/R injury from HS (5). We hypothesized that the biomarkers of metabolic alterations and organ injury typically occurring from I/R would be absent in both EU- and IBA-AGS. Here we show for the first time that during global I/R, the metabolic “fingerprint” of the I/R injury prone rats shifts while those of the AGS remains unaltered. Specifically, we were able to find that rats had metabolic alterations during global I/R that correspond to an increase in gluconeogenesis, glycolysis and anaerobic metabolism in addition to markers for organ dysfunction. In contrast, EU-AGS maintain stable concentrations of both hydrophobic and hydrophilic metabolites while IBA-AGS show increases in only two of the metabolites measured, tyrosine and alanine.

Metabolic Instability during HS in the I/R Injury Prone Species

Periods of ischemia followed by reperfusion are typically characterized by accelerated glucose consumption, glycolytic activity, and anaerobic oxidation of pyruvate to lactate (31, 32). Plasma metabolites in the rats were indicative of active carbohydrate metabolism and anaerobic metabolism (increased lactate, alanine). Glucose concentrations were not determined from the plasma spectra as presaturation to diminish the water signal also affected glucose peaks that are in close proximity. However, previous studies in our lab showed that glucose increased in the rat during I/R and for the three hours following reperfusion (5). Lactate is a well-established herald of poor prognosis after I/R injury (33, 34). It is one of four hydrophilic metabolites (glucose, glutamate, lactate, and β -hydroxybutyrate) that have been found by other ^1H -NMR metabolomic studies on I/R to have a high correlation with morbidity (35, 36). In our previous report, none of the rats subject to HS survived past 18 hours with most dying within three hours (5).

The I/R vulnerable rats also had a significant increase in oxaloacetate during HS. Increases in lactate, alanine, and oxaloacetate, all metabolites that can be converted to glucose via gluconeogenesis, are consistent with an increased flux of metabolites involved in gluconeogenesis in the rats under I/R conditions. Alanine, typically from muscle sources, is an important glyconeogenic substrate (32). As glycogen stores can also be a source of glucose, animals were fasted overnight (rats) or for ~20 hours (AGS) prior to the start of the experiments. Alanine may also be an indicator of dysfunctional lipid metabolism. However, no changes due to I/R were found in any of the groups in the hydrophobic metabolites (Table 3.1).

In contrast, IAB-AGS, but not EU-AGS, had increases in both glucose and lactate levels during I/R but not to the extent of rats (5). Avoidance of lactate build up is a key feature of ischemic preconditioning and has been shown to occur in during the winter season in hibernating ground squirrels, presumably contributing to their resistance to ischemic injury (5, 13, 25, 31, 37, 38). In this study, AGS of either season did not have increases in lactate or oxaloacetate, but

IBA-AGS did have an increase in alanine (EU-AGS had a trend towards increasing over sham, but it was not statistically significant).

The differences in hydrophilic metabolites reported here vary from those previously reported from $^1\text{H-NMR}$ analysis. Serkova et al. found that lactate and alanine concentrations were higher in summer versus IBA ground squirrels (13). However, that study was focused on monitoring metabolic changes in the liver during hibernation cycles in ground squirrels. The differences noted could be due to the tissue examined (liver versus plasma). It may also be due to the fact that the IBA animals used in the current study were completely euthermic and showed no signs (e.g. reduced body temperature) of entering into a torpor bout. In contrast, Serkova and colleagues specifically used IBA animals that dropped core body temperature and were beginning to enter torpor. Previous studies have shown proteomic and metabolomic differences between normothermic IBA animals and those who are in various stages of torpor (17-19). In this study, normothermic IBA animals were used, which had been handled gently ~ 20 hours prior to the start of the experiment to induce arousal. This was done for two reasons 1) to examine endogenous I/R protective mechanisms outside those of hypothermia, and 2) torpid animals have vastly depressed cardiovascular, respiratory, and metabolic rates that would confound not only the results, but also the experimental techniques (39, 40).

Others have shown a difference in hydrophobic metabolites between the summer and winter season in ground squirrels (13, 16). Surprisingly, in this study, there were no significant differences in lipophilic metabolites between the EU- and IBA-AGS at any time point or for either treatment (sham versus hemorrhagic shock; Table 3.1); although there was a trend for the IBA-AGS to have higher concentrations of the lipophilic metabolites than the EU-AGS. However, there was a difference between the two species with rat being lower than either AGS in either season overall (Table 3.1). Others have demonstrated that products of lipid metabolism are protective during I/R (41-44). As there was no significant change in any of the hydrophobic metabolites during the experiments within a group, it is unlikely that these contributed to

protective biochemical mechanisms in the AGS or injurious mechanism in the rat. There is a well documented difference in lipid metabolism between summer euthermic and winter ground squirrels (7, 12-22). With ^1H -NMR analysis, Serkova et al. found that only polyunsaturated fatty acids (PUFA) levels were greater in IBA versus summer animals. Most of the differences in the lipophilic metabolites were between summer and torpid animals (13). Epperson et al. found that cholesterol levels in plasma were higher in IBA versus summer animals (16). In the current study, the IBA animals tended to have higher cholesterol levels, but the difference was not significant. These discrepancies are most likely due to high variation between individuals in each group due to differences in interbout arousal duration and the how far along in the process each animal was. In both of the previous studies, IBA animals were studied at different timepoints within the IBA process (entrance into torpor and 3 hours after euthermic body temperature was reached), in a synchronized fashion. During each IBA, the animal goes through many dynamic physiological and biochemical alterations that are specific to the arousal/torpor cycle (16-18). The present study sought to examine metabolic mechanisms that differ between the summer and winter season, versus between the IBA and torpid state. As such, IBAs were not synchronized for experiments which might contribute to the higher variation in metabolite concentrations in this group.

Organ Damage Markers

In addition to flagging alterations in metabolic pathways and preferential fuel sources, increases in several of the metabolites are established to biomarkers for organ damage or dysfunction (25, 35, 37, 45, 46). The I/R injury prone rats had increases in lactate, tyrosine, and acetate, markers of hepatic injury, and creatinine, a gauge of renal dysfunction (Fig. 3.5). Lactate, a sign for anaerobic metabolism is also a marker of liver dysfunction and decreased aptitude of the liver's ability to clear circulating excess lactate from the blood stream (47). The same is true of tyrosine and acetate. Increases in amino acids, such as tyrosine, are indicative of decreased hepatic function (45) as is an increase in acetate, one of the major endpoints in

several hepatic metabolic pathways (37). When renal injury occurs, creatinine builds up in the blood stream due to the decreased waste-filtering capacity of the kidneys (37). Allantoin is another metabolite that is a marker for renal damage. In this study, allantoin levels differed between the treatment groups, but was not different between sham and hemorrhaged animals, perhaps pointing to some low-level renal dysfunction that was associated with the surgical procedure itself. These results agree with previous studies in our lab that have shown from standard blood chemistry analysis that after HS, rats have increased serum markers for liver (aspartate aminotransferase, and alanine aminotransferase) and kidney (creatinine) damage (5). Meanwhile, EU-AGS do not have an increase in any of the markers for organ damage after HS, while IBA-AGS showed an increase in tyrosine, a marker for liver dysfunction (45).

Previous studies have shown a difference in I/R tolerance between summer- and winter-season ground squirrels (6-8, 48). These experiments have been performed on various isolated tissue preps and have demonstrated resistance to I/R injury in winter, but not summer animals for liver, kidney, and small intestine. Similarly, only the brain of euthermic ground squirrels has been shown to be resistant to I/R injury (6-9). Our previous report detailed that both IBA and EU-AGS were resistant to multi organ failure after global I/R (5). As NMR is a relatively insensitive method requiring ~2-4 mM of metabolites for detection, some metabolic perturbations may need more time to build to sufficient concentrations (36). For example, allatonin has been found only to be distinguishable in the blood 24 hours after I/R (25)

For metabolomic analysis, plasma is considered the biofluid of choice (36) and is well established for the evaluation of the metabolic status of various organs (35). In principle it represents an “average” of the physiological biochemical status of the organism as it interacts with all of the various tissues and organs. However, hibernating animals have the ability to regulate blood flow away from peripheral regions during hibernation and arousal (49-52). Also, given the complexity of the hemodynamics during hemorrhage, there may be differential perfusion to various organs over the time course. In addition, EDTA was used as an

anticoagulant. EDTA produces NMR peaks that can obscure detection of certain metabolites (53). However, most of these metabolites can be quantified based on peaks in regions clear of the anticoagulant peaks (54).

Conclusion

Systemic I/R injury, such as occurs in HS, is a leading cause of death in trauma patients (35). Lack of oxygen and nutrients in conjunction with buildup of waste products during the ischemic period, followed by the inrush of oxygen and the generation of ROS during reperfusion induce metabolic alterations at the cellular level. By analyzing the metabolites and metabolic profile of a species known to be prone to I/R damage, both before and after I/R insult, we were able to establish biomarkers of metabolic perturbations and organ damage that precede HS-induced fatality. By then comparing the metabolic profile of the I/R-resistant AGS to these biomarkers, we discovered that, unlike the I/R injury prone rats, which experience distinct alterations in their metabolome during I/R, AGS are able to sustain their hydrophobic and hydrophilic metabolic profiles. Specifically, AGS resist changes to metabolites involved in carbohydrate turnover, anaerobic metabolism, and organ dysfunction. This information suggests that the underlying factors involved in I/R resistance in these animals is not based in the seasonal switch in energy source from carbohydrate in the summer to lipid in the winter but in the maintenance of metabolic homeostasis. The stability of their metabolome during the period of limited nutrient and oxygen availability indicates that they may have better metabolic regulatory and/or compensatory mechanisms not utilized by homothermic species. These metabolic mechanisms could provide potential therapeutic interventions to alleviate the metabolic imbalances and subsequent inflammatory response and organ failure associated with global I/R.

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Tables and Figures

Table 3.1. Average concentrations (mM plasma) and S.E.M. of metabolites for summer euthermic (EU) arctic ground squirrels, winter interbout arousal (IBA) arctic ground squirrels, and rats immediately before and three hours after hemorrhagic shock (HS) or sham (SHS). n=5-7. Group was defined as EU-AGS vs. IBA-AGS vs. rat. Treatment indicated sham vs. hemorrhagic shock. For all hydrophobic parameters, difference in group was IBA>rat. ANOVA analysis included sham surgery animals as a treatment (n=5-7). ANOVA value and significant differences ($p<0.05$, Tukey) are indicated. TAG, triacylglycerol; PUFA, Polyunsaturated fatty acids.

		EU-AGS		IBA-AGS		Rat		ANOVA	Interaction
		Before	After	Before	After	Before	After	p	or Main Effect
Hydrophilic metabolites by ¹ H-NMR									
Acetate	HS	0.075±0.018	0.086±0.011	0.090±0.009	0.097±0.009	0.165±0.011	0.028±0.023	0.0009	time x group x treatment
	SHS	0.079±0.011	0.099±0.006	0.075±0.006	0.082±0.011	0.153±0.010	0.170±0.023		
Alanine	HS	0.357±0.055	0.683±0.104	0.551±0.152	0.940±0.180	0.770±0.078	1.945±0.195	0.0007	time x group x treatment
	SHS	0.479±0.067	0.640±0.094	0.316±0.031	0.394±0.333	0.763±0.066	0.926±0.159		
Creatinine	HS	0.239±0.055	0.576±0.195	0.150±0.031	0.189±0.036	0.489±0.026	1.461±0.107	0.0329	time x group x treatment
	SHS	0.231±0.028	0.384±0.043	0.168±0.031	0.258±0.047	0.466±0.050	1.048±0.118		
Histidine	HS	0.073±0.010	0.090±0.008	0.070±0.007	0.082±0.010	0.059±0.007	0.064±0.019	0.0126	time x group x treatment
	SHS	0.086±0.008	0.094±0.011	0.085±0.007	0.067±0.018	0.068±0.003	0.115±0.007		
β-hydroxybutyrate	HS	0.258±0.068	0.355±0.059	1.861±0.688	0.928±0.265	0.898±0.043	0.789±0.054	0.0296	time x group x treatment
	SHS	0.288±0.067	0.517±0.066	2.954±1.118	3.605±1.111	0.938±0.118	0.963±0.209		
Lactate	HS	1.768±0.235	3.402±0.611	2.291±0.231	3.674±0.421	1.665±0.069	7.573±0.431	<0.0001	time x group x treatment
	SHS	2.497±0.406	3.124±0.379	2.123±0.105	2.189±0.231	1.972±0.204	3.537±0.500		

Table 3.1. Continued

Oxaloacetate	HS	0.113±0.027	0.130±0.017	0.134±0.014	0.146±0.013	0.247±0.017	0.420±0.034	0.0009	time x group x treatment
	SHS	0.118±0.016	0.149±0.009	0.112±0.010	0.123±0.017	0.229±0.015	0.255±0.035		
Tyrosine	HS	0.146±0.021	0.243±0.054	0.043±0.012	0.085±0.015	0.131±0.014	0.473±0.088	0.0001	time x group x treatment
	SHS	0.125±0.021	0.170±0.026	0.091±0.008	0.072±0.008	0.142±0.011	0.122±0.019		
Allantoinin	HS	0.124±0.013	0.187±0.020	0.696±0.391	0.475±0.118	0.081±0.008	0.409±0.023	0.0405	time x group
	SHS	0.232±0.070	0.248±0.042	0.239±0.030	0.189±0.033	0.096±0.010	0.252±0.040		
Betaine	HS	1.120±0.308	1.191±0.302	1.158±0.154	1.017±0.054	0.814±0.186	2.176±0.379	0.0079	time x group
	SHS	0.838±0.072	0.826±0.089	0.843±0.093	1.316±0.599	0.549±0.056	1.033±0.125		
Formate	HS	0.174±0.108	0.196±0.035	0.266±0.056	0.564±0.066	0.162±0.021	0.178±0.026	0.0001	time x group
	SHS	0.084±0.008	0.238±0.055	0.268±0.019	0.480±0.031	0.156±0.023	0.191±0.018		
Phenylalanine	HS	0.105±0.014	0.177±0.024	0.080±0.014	0.104±0.006	0.118±0.010	0.249±0.026	<0.0001	time x group
	SHS	0.094±0.009	0.127±0.009	0.073±0.008	0.062±0.005	0.109±0.003	0.162±0.009		
Pyruvate	HS	0.129±0.020	0.198±0.026	0.185±0.027	0.224±0.024	0.140±0.006	0.321±0.012	<0.0001	time x group
	SHS	0.127±0.018	0.195±0.026	0.108±0.010	0.141±0.013	0.156±0.018	0.257±0.031		
Valine	HS	0.316±0.046	0.446±0.031	0.295±0.055	0.278±0.034	0.341±0.021	0.396±0.033	0.0005	time x group
	SHS	0.332±0.034	0.408±0.035	0.206±0.019	0.233±0.013	0.309±0.028	0.283±0.013		
Choline	HS	0.725±0.213	0.651±0.064	0.535±0.073	0.535±0.032	0.289±0.022	0.380±0.032	<0.0001	group
	SHS	0.834±0.085	0.894±0.096	0.829±0.060	0.745±0.053	0.313±0.011	0.391±0.023		
Glutamine	HS	0.824±0.099	1.005±0.039	1.894±0.531	1.620±0.171	1.955±0.084	2.210±0.140	<0.0001	group
	SHS	0.899±0.084	1.034±0.068	1.061±0.061	1.215±0.040	1.752±0.143	2.048±0.060		

Table 3.1. Continued

Glycine	HS	0.670±0.164	1.055±0.049	1.537±0.271	1.372±0.107	1.408±0.103	1.649±0.077	0.0021	group
	SHS	0.696±0.161	0.899±0.149	0.719±0.144	1.135±0.283	1.123±0.193	1.403±0.157		
Threonine	HS	0.475±0.114	0.542±0.082	0.329±0.051	0.319±0.050	0.399±0.070	0.658±0.085	0.0122	group
	SHS	0.603±0.089	0.662±0.095	0.497±0.065	0.325±0.136	0.469±0.047	0.512±0.051		
Isoleucine	HS	0.160±0.015	0.227±0.012	0.399±0.204	0.272±0.061	0.186±0.013	0.188±0.015	n/a	
	SHS	0.194±0.026	0.232±0.024	0.119±0.019	0.137±0.012	0.163±0.015	0.141±0.005		
Leucine	HS	0.390±0.047	0.509±0.029	0.778±0.346	0.569±0.108	0.377±0.022	0.433±0.032	n/a	
	SHS	0.437±0.057	0.489±0.039	0.330±0.045	0.331±0.022	0.349±0.028	0.317±0.012		
Hydrophobic metabolites by ¹ H-NMR									
TAG	HS	2.889±0.811	3.858±0.753	14.737±4.901	27.610±12.470	1.446±0.283	1.044±0.158	n/a	
	SHS	12.421±7.879	10.259±3.442	9.348±1.859	9.166±2.675	1.773±0.411	2.120±0.601		
Cholines	HS	3.992±1.084	5.244±0.799	12.027±2.789	14.520±6.765	1.930±0.240	1.398±0.221	0.0005	group
	SHS	8.708±2.991	10.368±2.635	10.824±2.450	10.620±3.157	2.119±0.512	2.704±0.782		
PUFA	HS	6.482±1.914	8.055±1.092	12.278±2.417	17.120±7.362	7.840±1.403	5.217±0.658	n/a	
	SHS	13.126±5.265	15.178±3.400	10.684±2.931	10.937±2.931	9.460±2.440	12.453±4.016		
Total Triglycerides	HS	24.433±6.322	33.194±5.831	101.047±26.99 5	168.324±71.70 7	16.813±2.778	11.310±1.180	0.0004	group
	SHS	77.615±40.553	73.733±21.553	70.655±13.842	69.278±19.516	21.048±4.796	24.020±6.859		
Cholesterol	HS	3.156±0.775	4.144±0.719	10.115±2.288	13.042±5.152	2.383±0.412	1.716±0.212	0.0012	group
	SHS	6.500±2.259	7.594±1.950	8.118±1.6411	7.847±2.092	3.105±0.820	3.999±1.303		

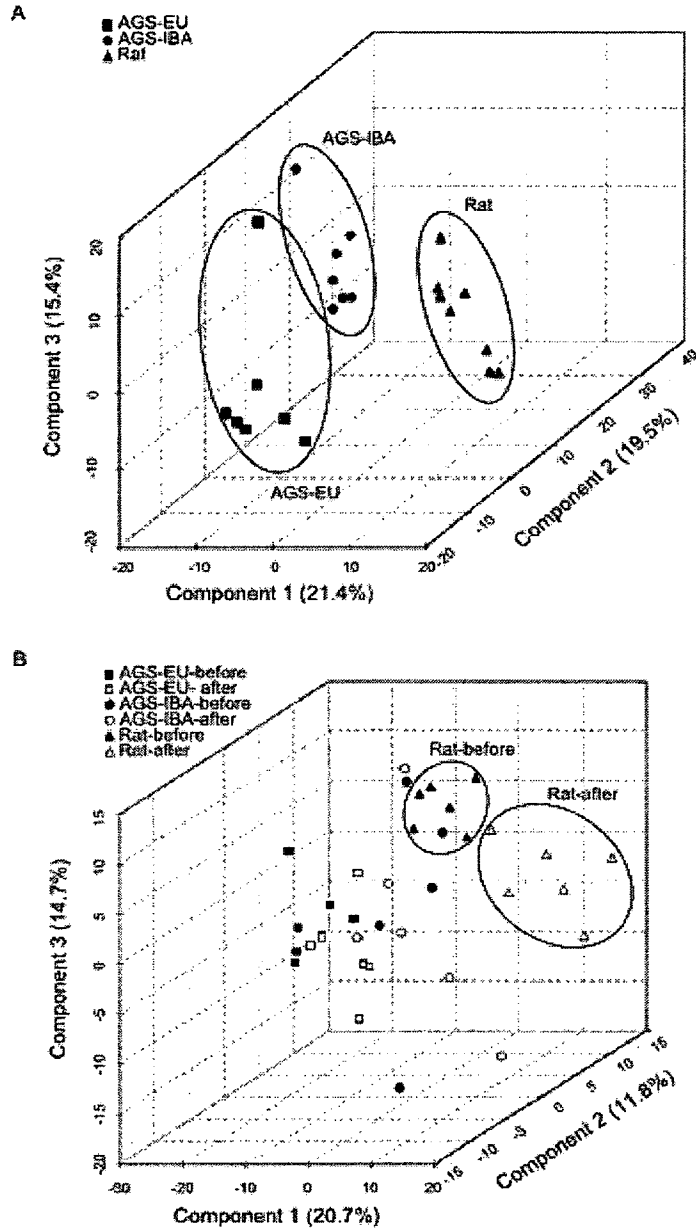


Figure 3.1. A: Hydrophilic metabolic profiles for naïve summer euthermic and winter IBA AGS formed distinct clusters separate from each other and the naïve rats. B: I/R injury-prone rats displayed different hydrophilic metabolic profiles before and three hours after hemorrhagic shock. No such clustering was evident for the AGS in either hibernation season. PLS-DA analysis of ^1H -NMR of naïve plasma samples (A) or samples from before and after hemorrhagic shock (B).

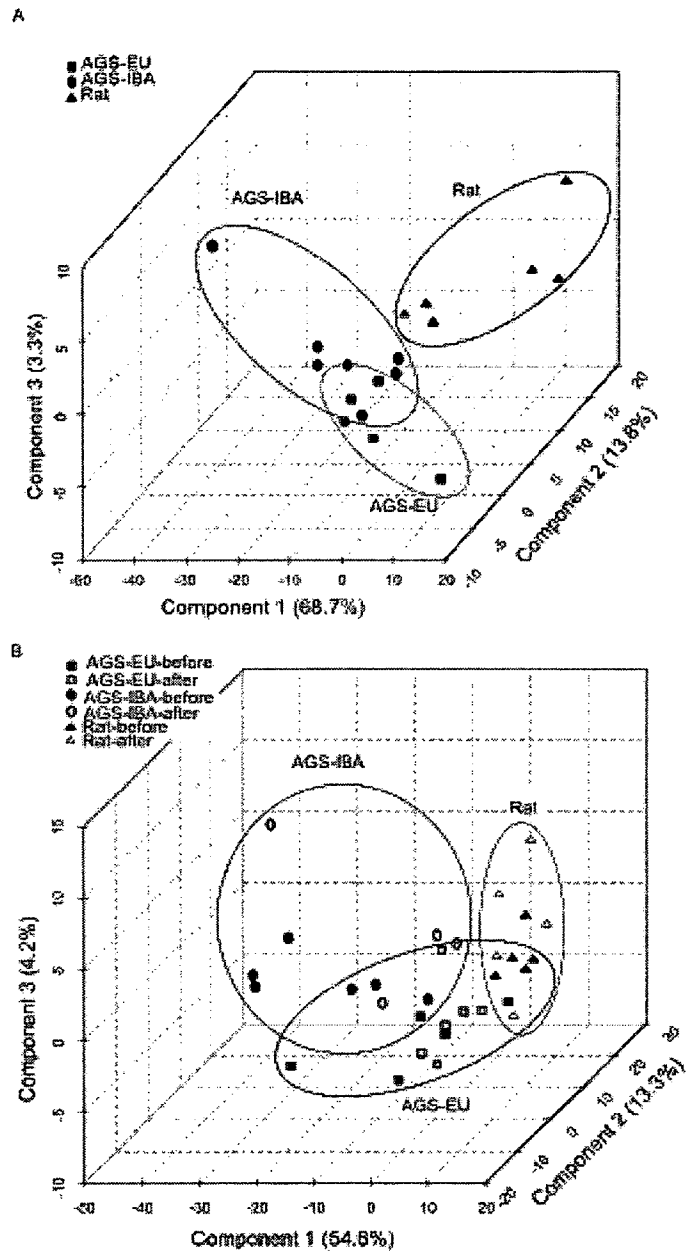


Figure 3.2. A: Lipid metabolic profiles for naïve rat were distinct from summer euthermic and winter IBA AGS while both season of AGS overlapped via PLS-DA analysis of the ^1H -NMR spectra. B: Interbout arousal arctic ground squirrels were distinct from I/R prone rats while euthermic arctic ground squirrels overlapped both rats and interbout arousal squirrels in PLS-DA analysis of ^1H -NMR lipid-extracted plasma samples before and after hemorrhagic shock.

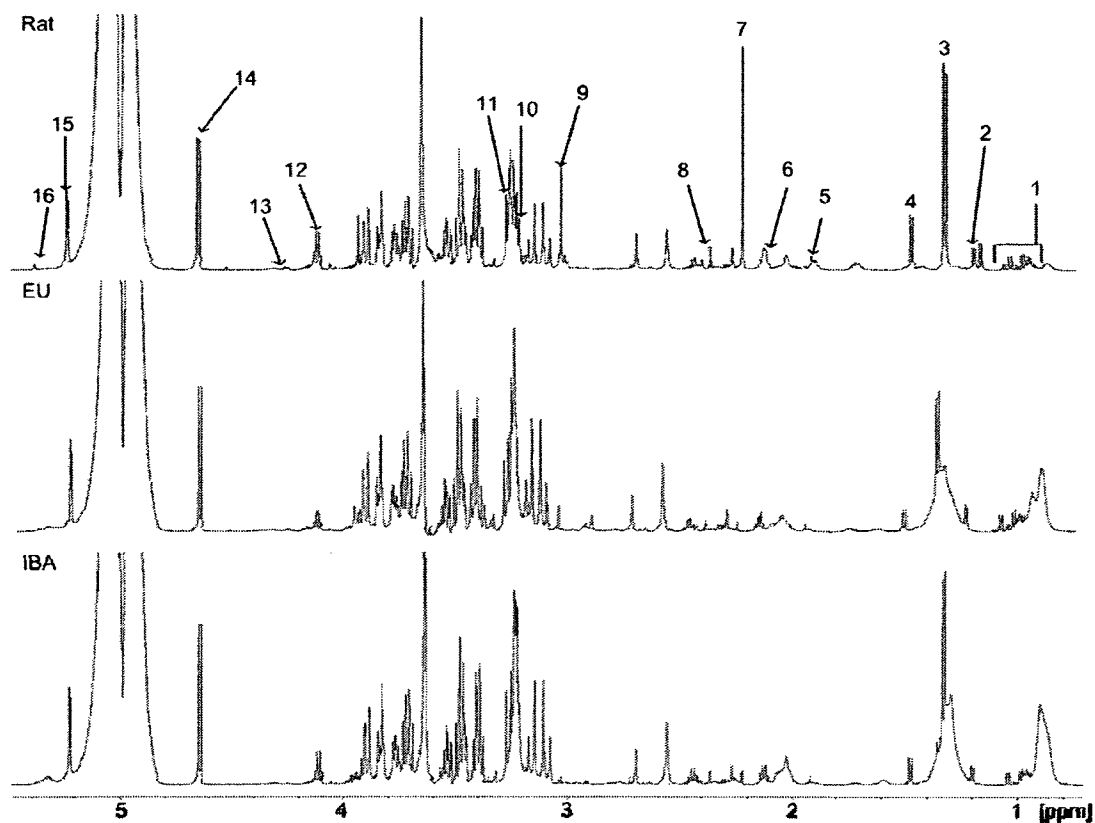


Figure 3.3. Representative ^1H -NMR spectra of naïve plasma samples from rats, summer euthermic (EU) arctic ground squirrel,, and winter interbout arousal (IBA) arctic ground squirrels. NMR peak assignments: 1, valine+leucine+isoleucine; 2, β -hydroxybuterate; 3, lactate + threonine; 4, alanine; 5, acetate; 6, glutamine; 7, acetoacetate; 8, pyruvate; 9, creatine; 10, choline; 11, betaine; 12, lactate; 13, threonine; 14, β -glucose; 15, α -glucose; 16, allantoin.

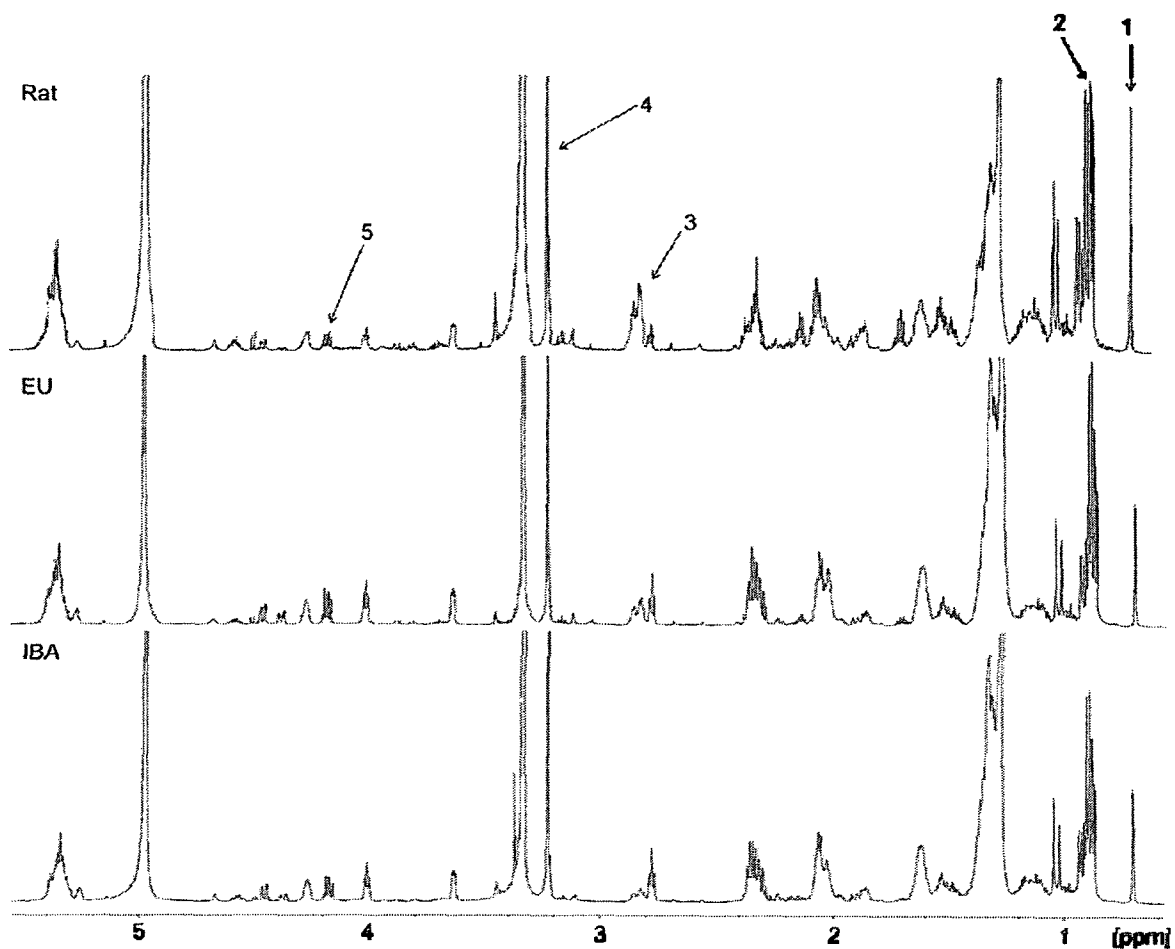


Figure 3.4. Representative ^1H -NMR spectra of naïve plasma lipid extract samples from rats, summer euthermic (EU) arctic ground squirrel, and winter interbout arousal (IBA) arctic ground squirrels. NMR peak assignments: 1, cholesterol; 2, total triglycerides; 3, PUFA, Polyunsaturated fatty acids; 4, cholines; 5, TAG, triacylglycerol.

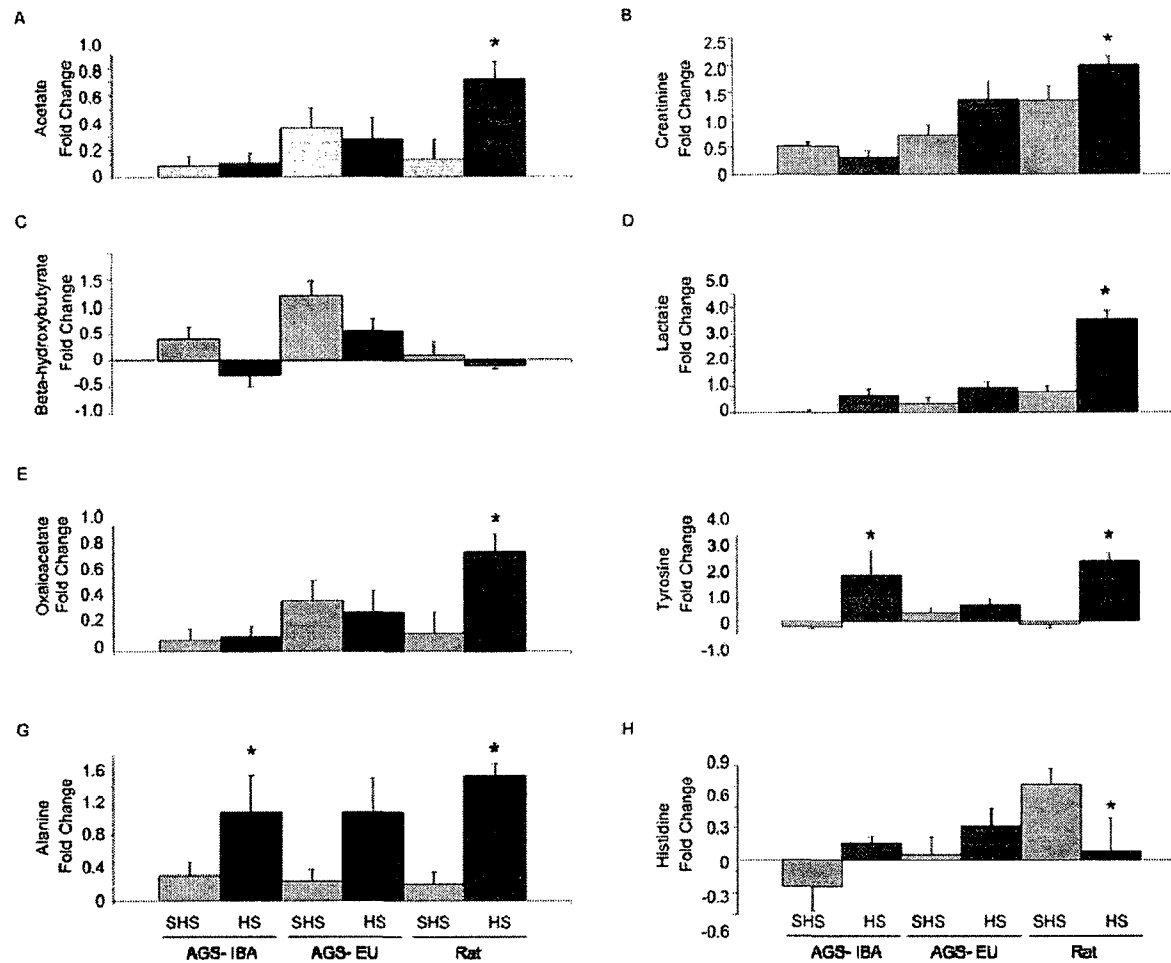


Figure 3.5. Fold change in circulating metabolites before and after sham hemorrhagic shock (SHS) or hemorrhagic shock (HS) in winter interbout arousal (IBA) arctic ground squirrels, summer euthermic (EU) arctic ground squirrels, and rats. Data presented as mean \pm S.E.M.. Asterisks indicate $p < 0.05$ vs. SHS of same group, Tukey test. $n=6-8$ for all groups. Concentrations of metabolites are shown in Table 3.1.

Supplemental Table 3.1. Characteristics of AGS undergoing HS during the winter (IBA) season.

Animal number	09-56	08-83	08-40	08-68	10-05	10-48
Season	Winter					
Age	Adult	Adult	Adult	Adult	Adult	Adult
Sex	Female	Female	Female	Male	Female	Female
Mass (g)	862	1123	1044	1056	559	721
First day of spontaneous torpor	18-Aug-10	18-Aug-10	17-Aug-10	4-Aug-10	29-Aug-10	2-Oct-10
Experiment day	7-Jan-11	11-Jan-11	24-Jan-11	27-Jan-11	17-Feb-11	18-Feb-11
No. of spontaneous torpor bouts prior to HS	11	16	14	14	13	13
Ave length of previous 3 torpor bouts (days)	15	11	11	14	18	18
Day in bout	6	4	4	4	2	11
Tb at induced arousal (°C)	3	3.3	2.9	3	4	4.7
Blood volume removed (% total)	16	15	22	24	28	37

Supplemental Table 3.2. Characteristics of AGS undergoing HS during the summer (euthermic) season.

Animal number	10-26	10-46	10-06	10-44	09-59	09-54
Season	Summer					
Age	Adult	Adult	Adult	Adult	Adult	Adult
Sex	Male	Male	Female	Male	Female	Female
Mass (g)	856	549	580	619	591	673
Last day of torpor during previous season	26-Jan-11	10-Feb-11	24-Feb-11	23-Feb-11	12-Mar-11	5-Apr-11
Experiment day	9-Jun-11	14-Jun-11	23-Jun-11	24-Jun-11	8-Jul-11	14-Jul-11
Blood volume removed (% total)	22	28	62	40	34	38

Supplemental Table 3.3. Characteristics of AGS undergoing SHS during the winter (IBA) season.

Animal number	08-51	09-86	08-97	10-09	10-24	10-28	10-33	10-12
Season	Winter							
Age	Adult	Adult	Adult	Adult	Adult	Adult	Adult	Adult
Sex	Female	Male	Female	Female	Male	Male	Male	Female
Mass (g)	671	704	904	454	738	729	731	438
First day of spontaneous torpor	7-Aug-10	18-Aug-10	17-Aug-10	29-Aug-10	7-Dec-10	16-Oct-10	1-Nov-10	16-Sep-10
Experiment day	21-Jan-11	18-Jan-11	19-Jan-11	28-Jan-11	3-Feb-11	4-Feb-11	11-Feb-11	23-Feb-11
No. of spontaneous torpor bouts prior to SHS	12	13	11	12	5	10	8	11
Ave length of previous 3 torpor bouts (days)	10	14	18	14	14	17	14	14
Day in bout	3	5	13	8	7	6	7	5
Tb at induced arousal (°C)	3.1	not taken	3.3	4	3.7	4.2	3.8	3.7

Supplemental Table 3.4. Characteristics of AGS undergoing SHS during the summer (euthermic) season.

Animal number	09-76	09-83	10-16	10-27	10-14	09-68	10-30
Season	Summer						
Age	Adult	Adult	Adult	Adult	Adult	Adult	Adult
Sex	Female	Male	Male	Male	Female	Female	Female
Mass (g)	1036	554	464	658	617	833	446
Last day of torpor during previous season	10-Nov-10	10-Feb-11	20-Feb-11	09-Feb-11	28-Feb-11	5-Jan-11	27-Feb-11
Experiment day	7-Jun-11	10-Jun-11	16-Jun-11	21-Jun-11	28-Jun-11	29-Jun-11	15-Jul-11

Supplemental Table 3.5. Average concentrations (mM plasma) \pm S.E.M. of metabolites for naïve summer euthermic (EU) arctic ground squirrels, winter interbout arousal (IBA) arctic ground squirrels, and rats. TAG, triacylglycerol; PUFA, Polyunsaturated fatty acids. n=6-7.

	Naïve EU-AGS	Naïve IBA-AGS	Naïve Rat
Hydrophilic metabolites by $^1\text{H-NMR}$			
Acetate	0.110 \pm 0.009	0.078 \pm 0.005	0.190 \pm 0.013
Alanine	0.454 \pm 0.086	0.346 \pm 0.040	0.625 \pm 0.040
Allantoin	0.218 \pm 0.073	0.232 \pm 0.052	0.138 \pm 0.019
Betaine	1.004 \pm 0.151	0.877 \pm 0.081	0.570 \pm 0.043
Choline	0.646 \pm 0.053	0.726 \pm 0.045	0.462 \pm 0.034
Creatinine	0.217 \pm 0.019	0.168 \pm 0.027	0.225 \pm 0.012
Formate	0.199 \pm 0.013	0.152 \pm 0.008	0.141 \pm 0.025
Glutamine	1.118 \pm 0.099	1.098 \pm 0.093	2.022 \pm 0.045
Glycine	0.712 \pm 0.062	0.839 \pm 0.072	0.735 \pm 0.066
Histidine	0.076 \pm 0.007	0.086 \pm 0.006	0.066 \pm 0.006
β -hydroxybutyrate	0.442 \pm 0.095	0.668 \pm 0.240	0.161 \pm 0.016
Isoleucine	0.199 \pm 0.053	0.130 \pm 0.019	0.127 \pm 0.007
Lactate	1.808 \pm 0.219	1.943 \pm 0.144	2.227 \pm 0.222
Leucine	0.441 \pm 0.099	0.359 \pm 0.004	0.273 \pm 0.011
Oxaloacetate	0.165 \pm 0.013	0.118 \pm 0.007	0.286 \pm 0.019
Phenylalanine	0.074 \pm 0.009	0.063 \pm 0.008	0.090 \pm 0.009
Pyruvate	0.148 \pm 0.008	0.108 \pm 0.010	0.146 \pm 0.009
Threonine	0.433 \pm 0.047	0.440 \pm 0.058	0.608 \pm 0.037
Tyrosine	0.156 \pm 0.024	0.083 \pm 0.011	0.116 \pm 0.014
Valine	0.308 \pm 0.046	0.214 \pm 0.014	0.210 \pm 0.005
Hydrophobic metabolites by $^1\text{H-NMR}$			
TAG	10.687 \pm 6.183	11.682 \pm 3.544	1.903 \pm 0.340
Cholines	6.352 \pm 1.045	11.113 \pm 3.038	2.533 \pm 0.926
PUFA	11.939 \pm 2.286	12.248 \pm 3.498	6.465 \pm 1.068
Total Triglycerides	64.594 \pm 30.525	84.074 \pm 22.556	15.428 \pm 2.157
Cholesterol	4.968 \pm 1.210	9.417 \pm 2.144	1.904 \pm 0.223

Conclusion

Ischemia/reperfusion injury and the subsequent development of MOF poses a serious medical complication to trauma patients. Obligate hibernators such as ground squirrels may hold insight into possible interventions to ameliorate the downstream organ damage from I/R injury. To date, researchers have explored several of the mechanisms that may contribute to the ability of these animals to repeatedly tolerate I/R. The present work demonstrates the both summer and winter season AGS are resistant to organ damage, systemic inflammation, and MOF after I/R and this resistance is not dependent on their ability to cool and become hypothermic during insult. In addition, metabolomic analysis indicates that the ability of these animals to resist I/R injury may stem from an intrinsic ability to maintain metabolic homeostasis during I/R. The stability of their metabolome during I/R may be due to metabolic regulatory and/or compensatory mechanisms not available and/or utilized by non-hibernating species. Determining the basis of the metabolic stability during I/R observed in the AGS may provide potential therapeutic interventions. Such interventions could be imperative during human I/R states that would alleviate the production and/or accumulation of detrimental metabolites, the subsequent systemic inflammatory response, and ultimately MOF and death.

Appendix A. Supplemental Data

Cardiac Arrest

The following are data obtained during the course of the CA experiments but did not display a difference due to ischemia/reperfusion injury. Sham animals underwent the same surgical procedure as the CA animals but were not asphyxiated and did not experience CA. Analysis of circulating metabolites, blood chemistries and complete blood count was limited after the CA procedure due to the high incidence of the chronic cannulas becoming obstructed during the course of the observation time and yielding insufficient sample size for analysis (blood chemistries). Before and one hour after CA have $n=4-6$, while between 24-144 hours after had $n=0-5$, depending on the parameter being assayed and the timepoint. All values shown as $\text{mean} \pm \text{S.E.M.}$ Statistical analysis via ANOVA followed by Tukey post hoc was performed only when noted with significance at $p < 0.05$.

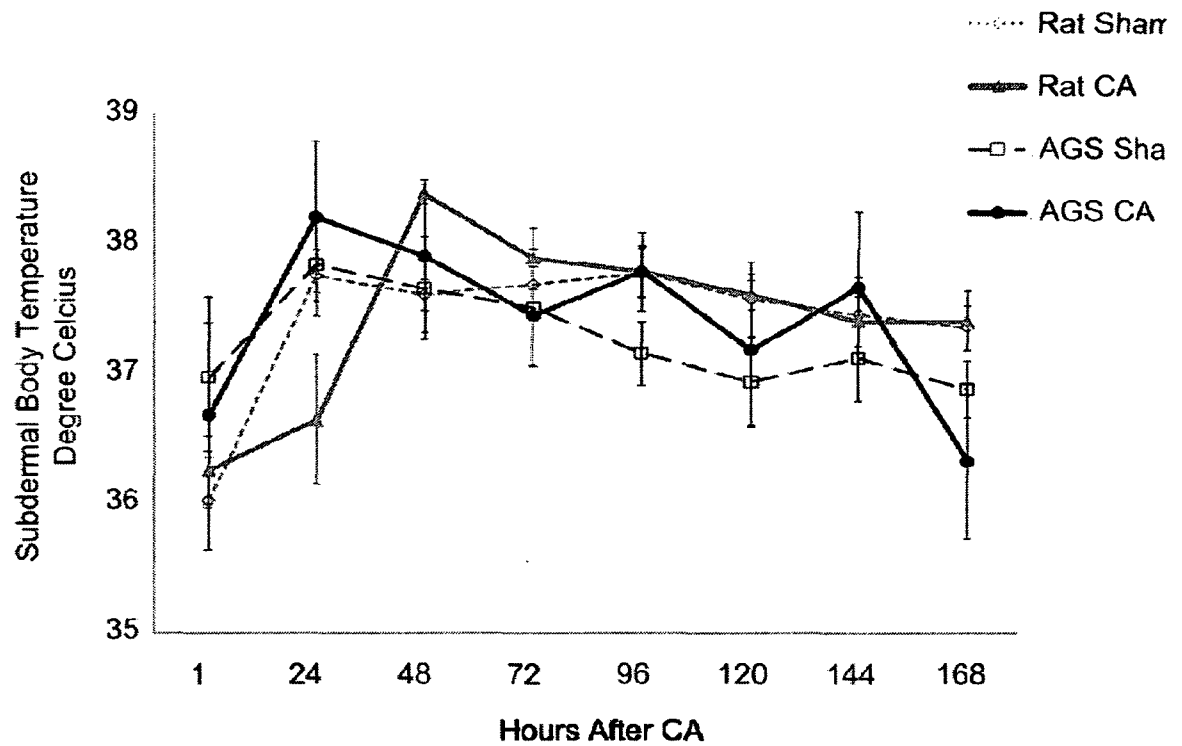
Body Temperature

Figure A1. Subdermal body temperatures did not show differences between groups. n=3-6. Naïve values were not obtained during the study

Neuroscores

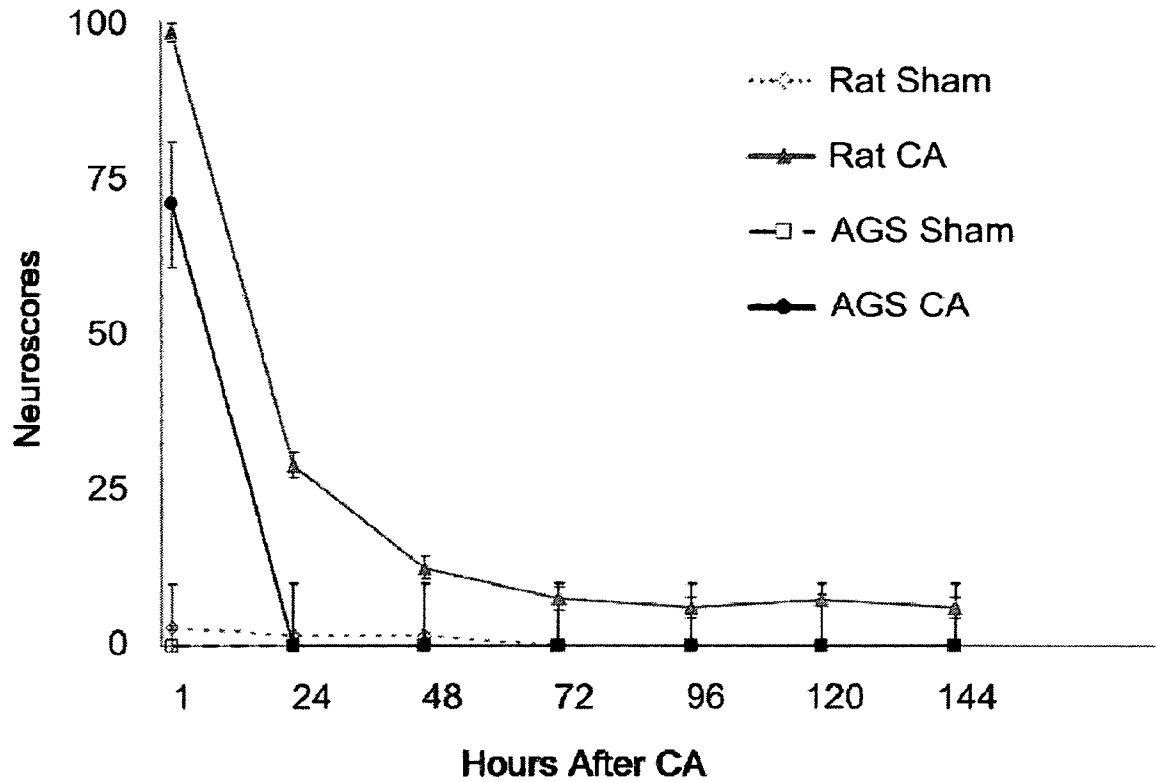


Figure A2. AGS had less neurological impairment 1- and 24 hours after CA than did rats as shown by lower neuroscores (a score of 0 denoting no deficit). By 48 hours after CA, impairment in the rats consisted of hind leg immobility. This may have been due to local nerve damage during cannulation and not an effect of the CA-induced global I/R. $n=5-6$. No naïve data were obtained

Metabolites

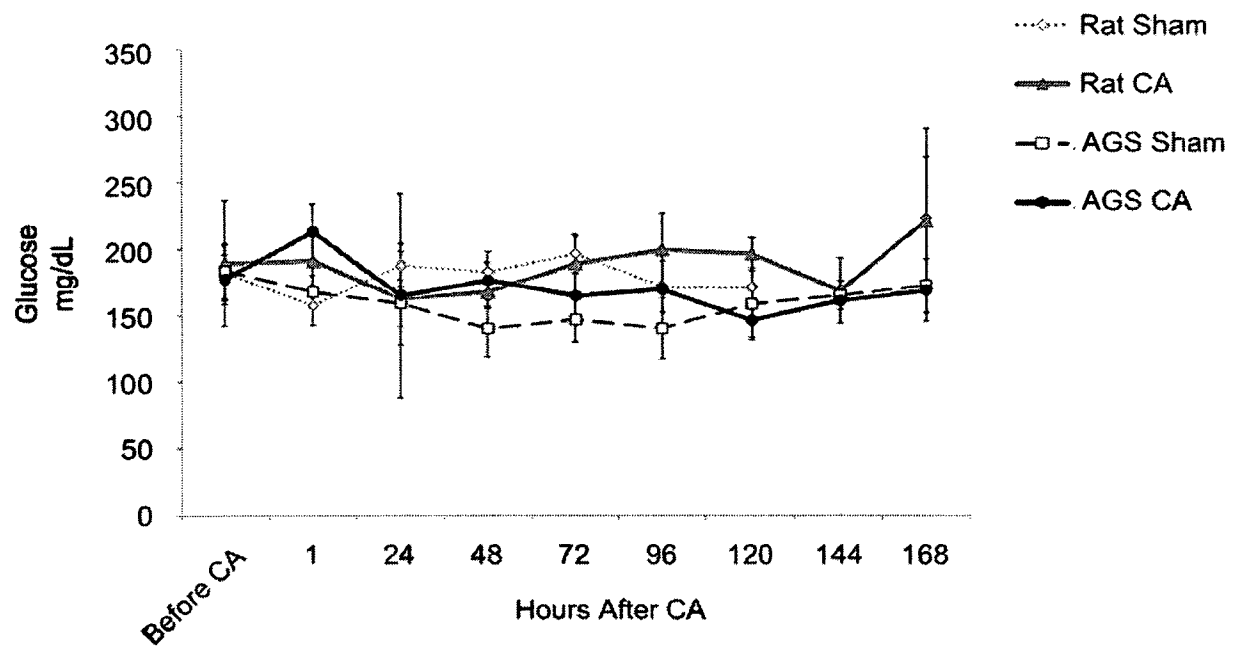


Figure A3. Blood glucose levels were similar for rats and AGS regardless of treatment. $n=0-5$. Naïve values were 205.67 ± 7.78 mg/dL for rats and 162.29 ± 10.94 mg/dL for euthermic, summer season AGS. Data given as mean \pm S.E.M.

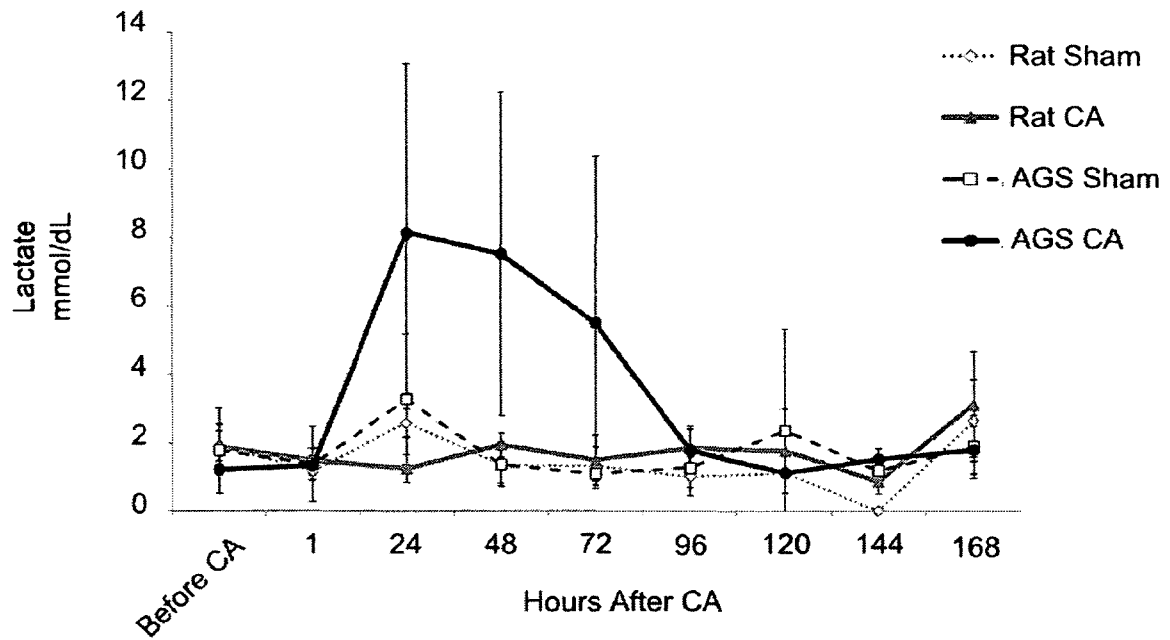


Figure A4. Blood lactate levels were not statistically different for rats and AGS at any timepoint regardless of treatment. $n=0-5$. Naïve values were 1.92 ± 0.20 mmol/L for rats and 1.66 ± 0.29 mmol/L for euthermic, summer season AGS. Data given as mean \pm S.E.M. Statistical analysis via ANOVA followed by Tukey post hoc.

Blood Chemistries

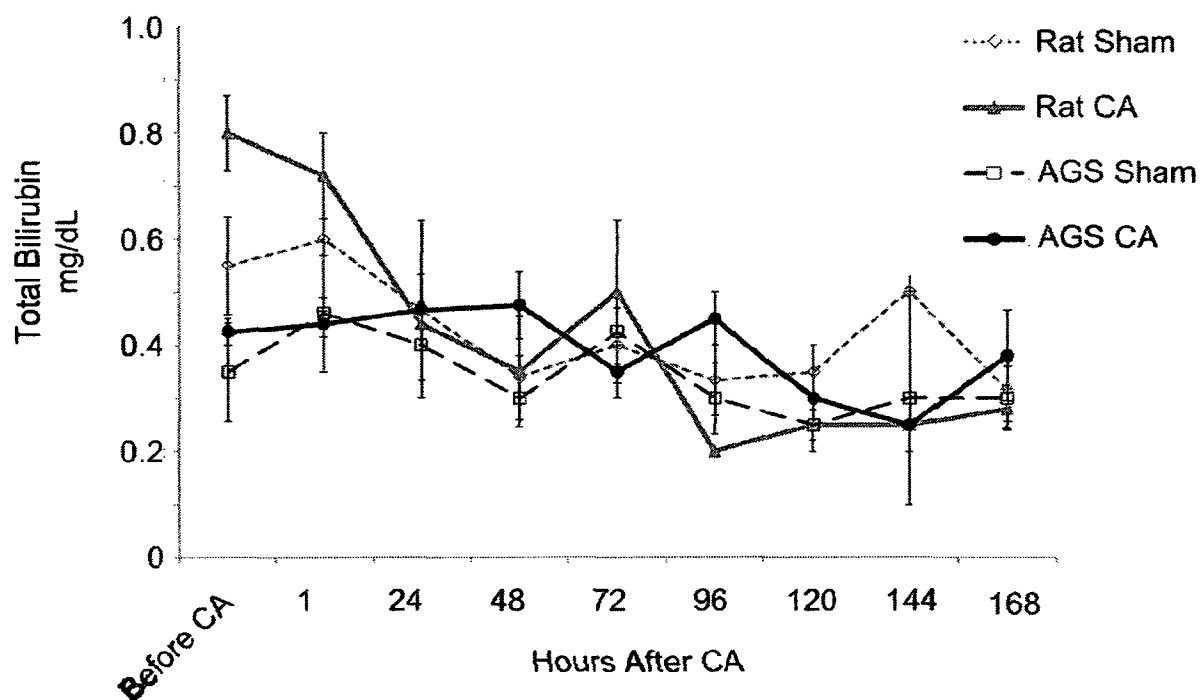


Figure A5. Total bilirubin levels showed no difference for rats and AGS at any timepoint regardless of treatment. $n=2-6$. Naïve values were 0.47 ± 0.04 mg/dL for rats and 0.39 ± 0.07 mg/dL for euthermic, summer season AGS. Data given as mean \pm S.E.M.

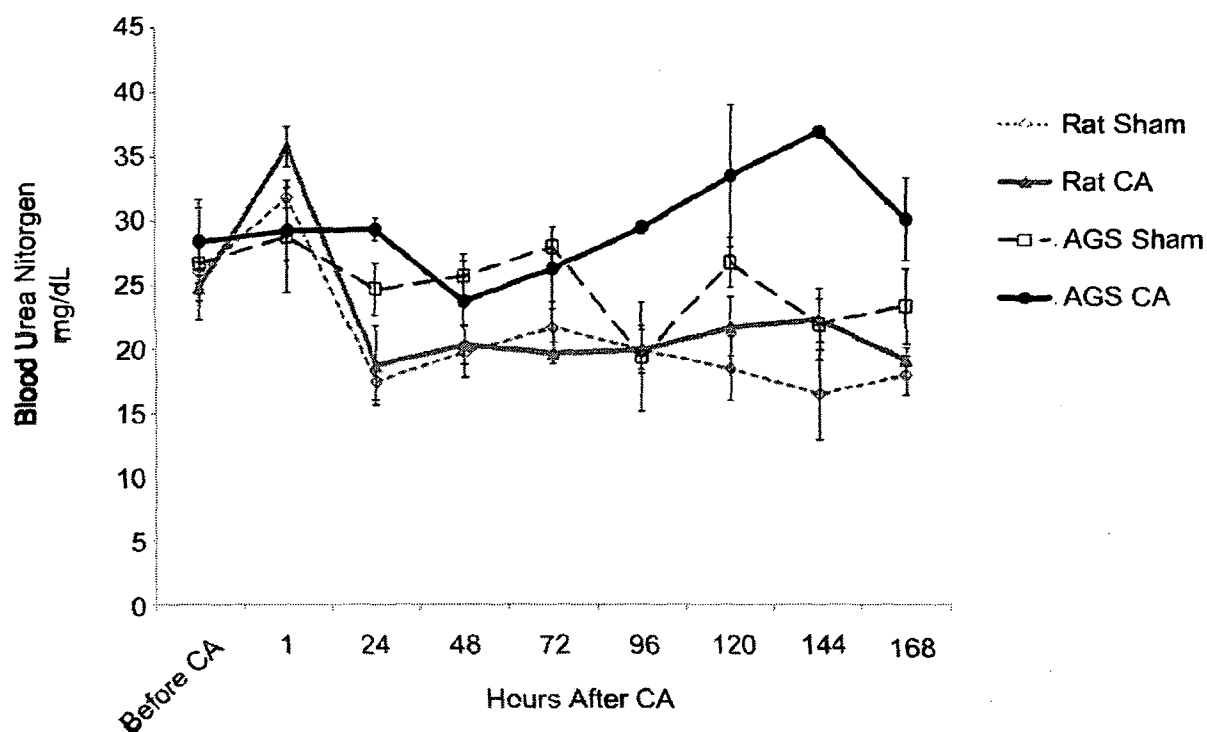


Figure A6. Blood urea nitrogen quantities showed no difference for rats and AGS at any timepoint regardless of treatment. $n=1-6$. Naïve values were 18.71 ± 0.84 mg/dL for rats and 27.00 ± 2.87 mg/dL for euthermic, summer season AGS. Data given as mean \pm S.E.M..

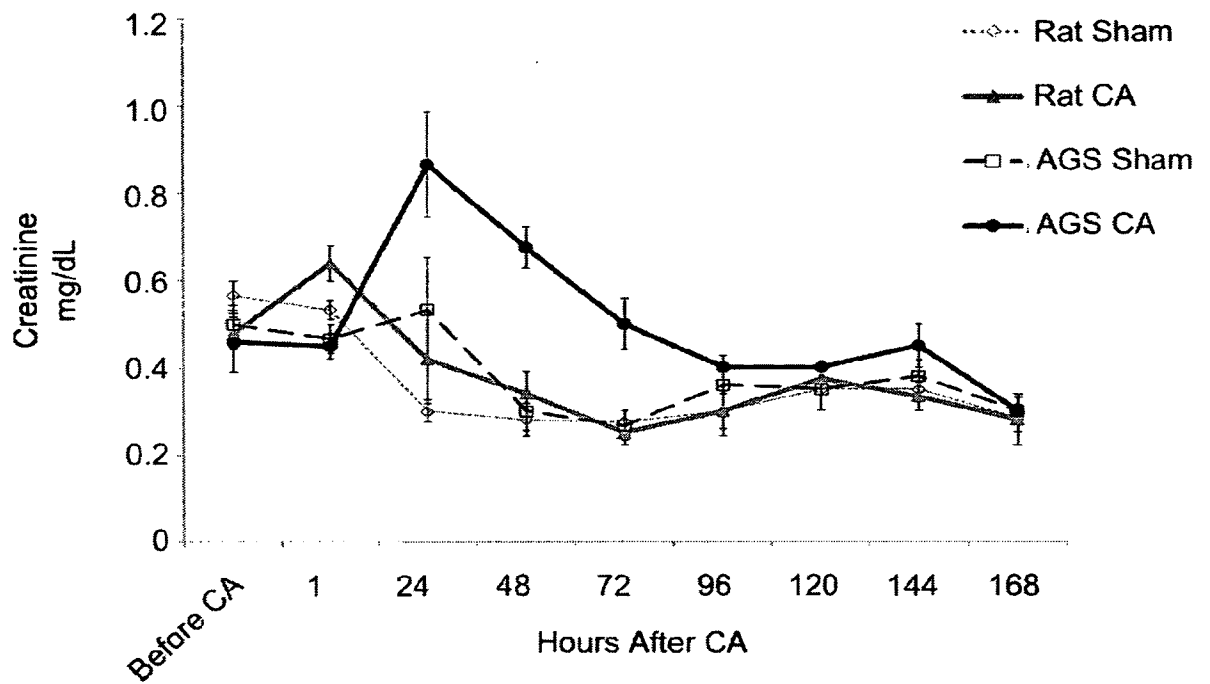


Figure A7. Circulating creatinine appeared to increase 24 hours after CA for AGS (n=3 for CA and sham AGS at the 24 hour timepoint). Levels were comparable to shams and rats at time of tissue harvested and no histopathology was found in the kidney. n=2-6. Naïve values were 0.37 ± 0.02 mg/dL for rats and 0.39 ± 0.03 mg/dL for euthermic, summer season AGS. Data given as mean \pm S.E.M..

Complete Blood Count

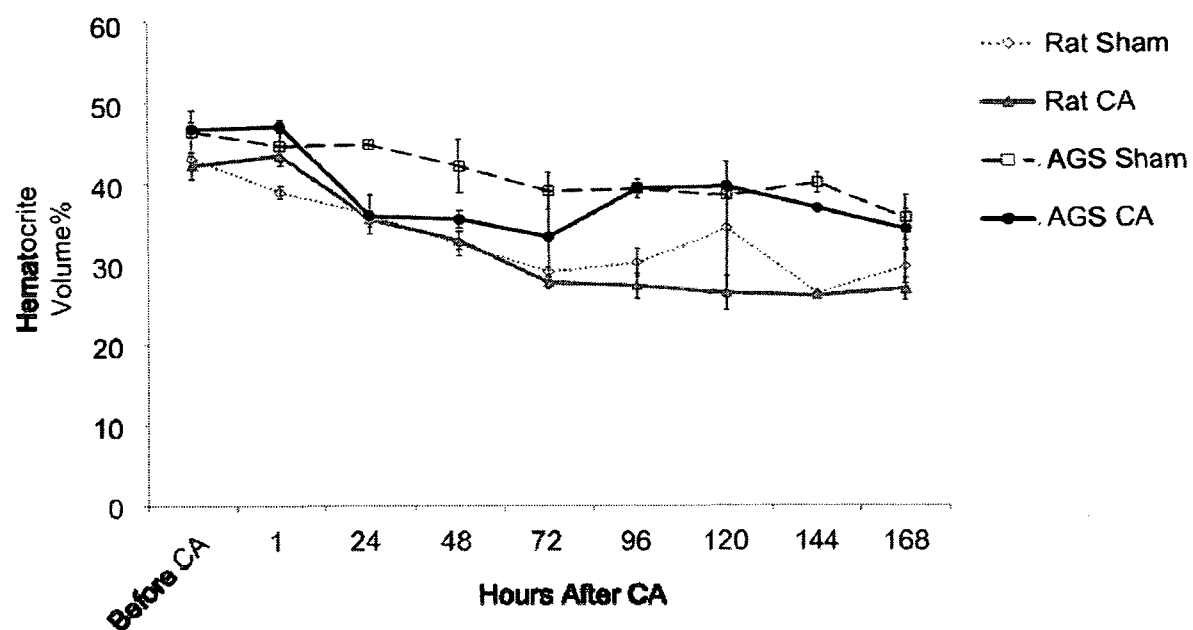


Figure A8. Hematocrit decreased for all treatment groups over the observation period. This is most likely due to daily blood sampling. $n=1-6$. Naïve values were 42.29 ± 1.35 Volume% for rats and 36.94 ± 0.92 Volume% for euthermic, summer season AGS. Data given as mean \pm S.E.M..

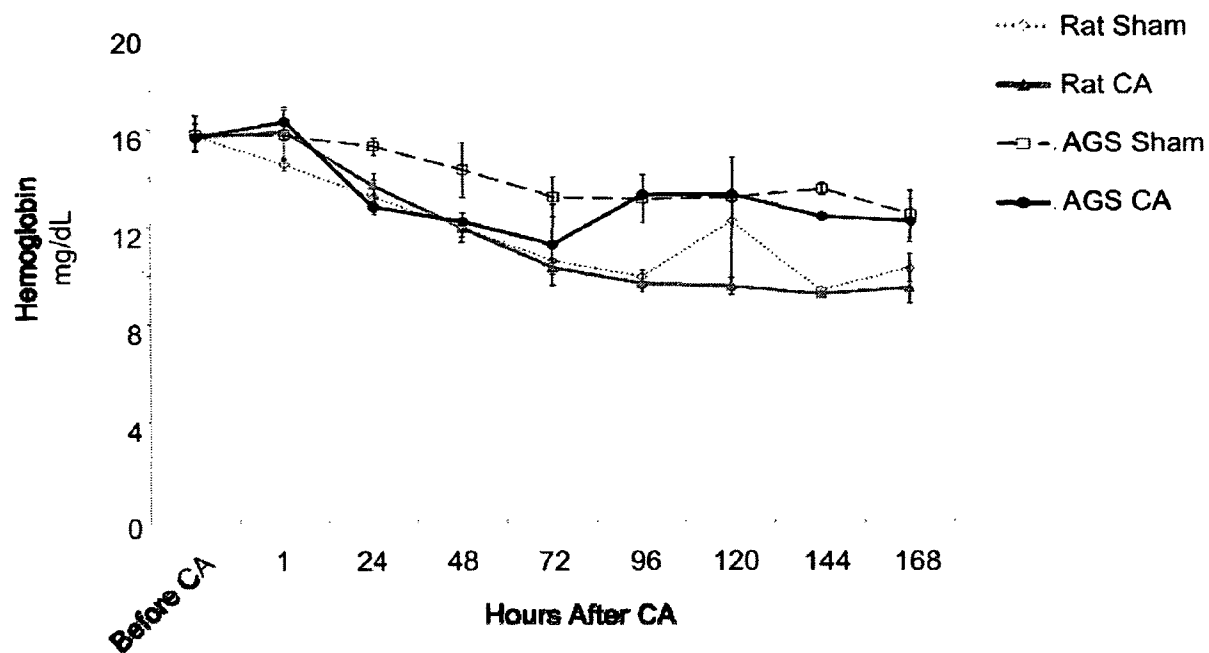


Figure A9. Hemoglobin levels decreased for all treatment groups over the observation period. This is most likely due to daily blood sampling. $n=1-6$. Naïve values were 14.96 ± 0.42 mg/dL for rats and 14.81 ± 0.51 mg/dL for euthermic, summer season AGS. Data given as mean \pm S.E.M..

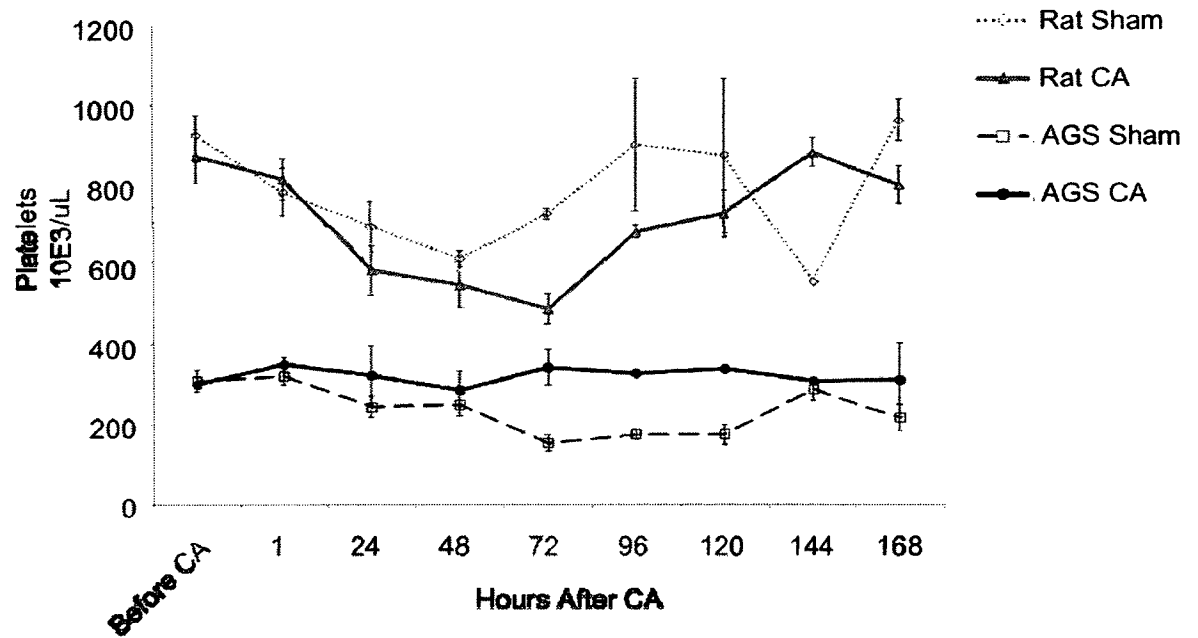


Figure A10. Platelet levels showed a difference between rats and AGS. $n=1-6$. Naïve values were 794.38 ± 95.95 $10^3/\mu\text{L}$ for rats and 266.71 ± 28.14 $10^3/\mu\text{L}$ for euthermic, summer season AGS. Data given as mean \pm S.E.M..

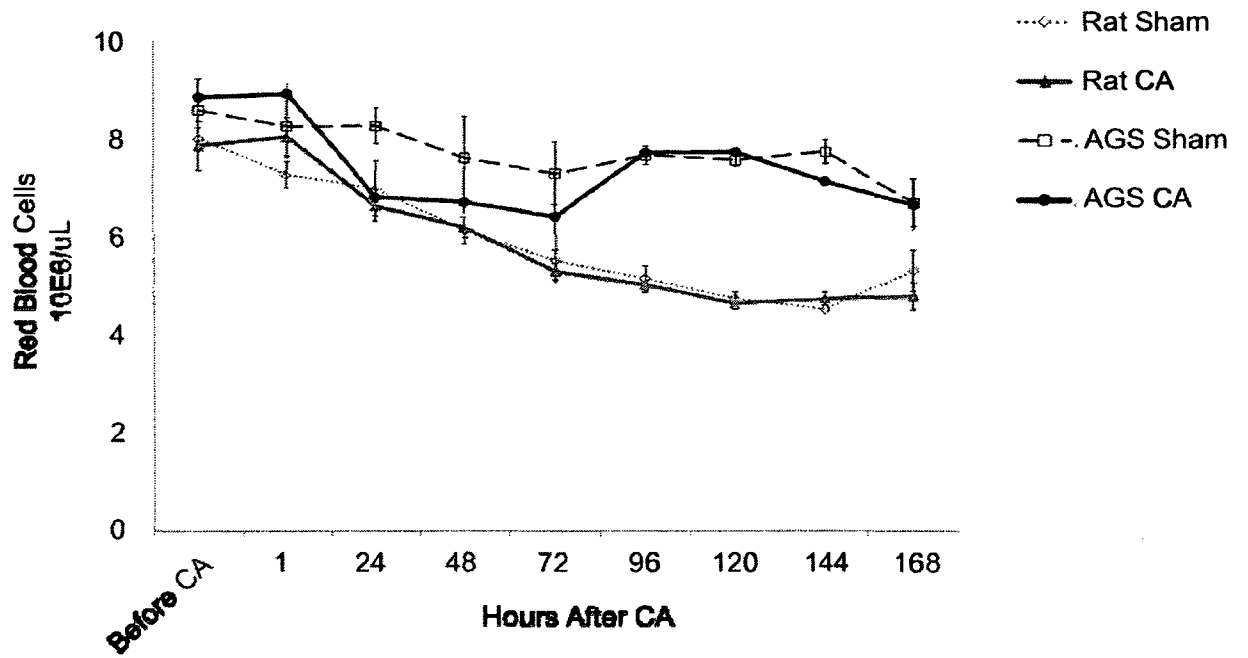


Figure A11. Red blood cell counts fell more noticeably for rats versus AGS. This could be due to daily blood sample volume being the same for each species while the AGS had a larger mean body mass. $n=1-6$. Naïve values were 8.2 ± 0.31 $10^6/\mu\text{L}$ for rats and 7.81 ± 0.11 $10^6/\mu\text{L}$ for euthermic, summer season AGS. Data given as mean \pm S.E.M..

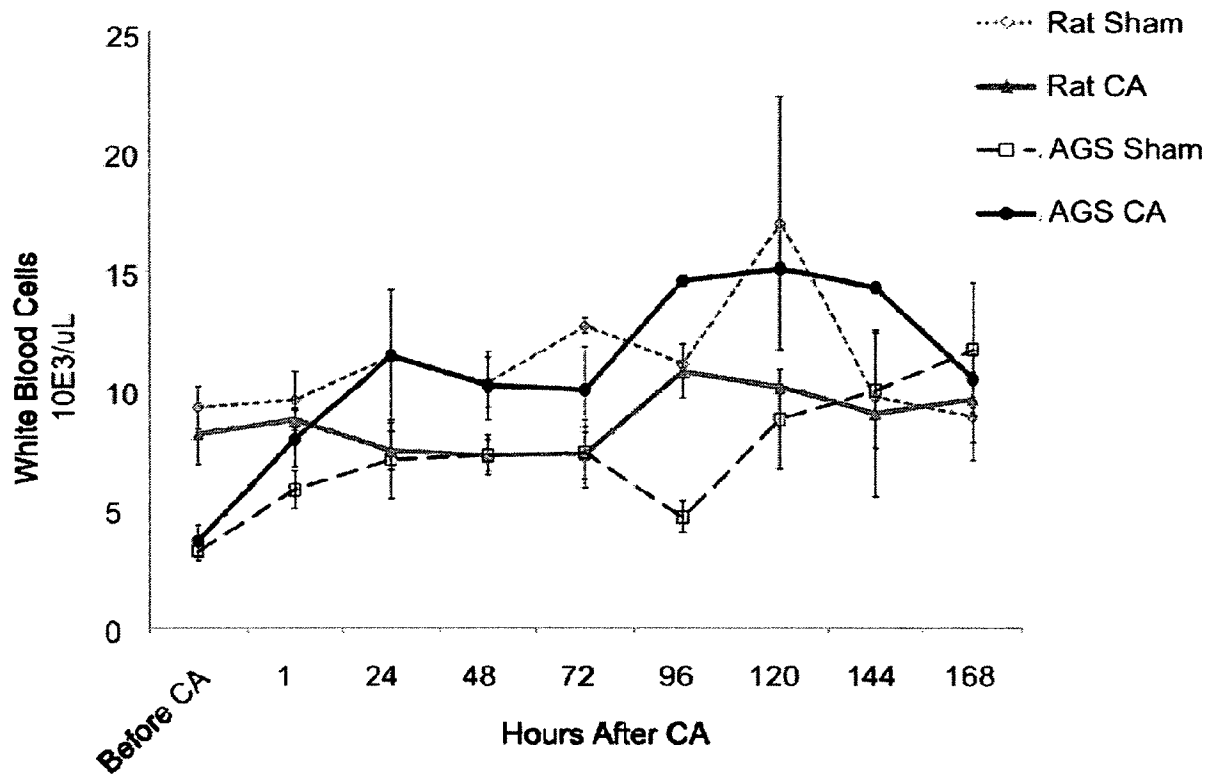


Figure A12. White blood cell counts during the seven days following treatment. $n=1-6$. Naïve values were 11.89 ± 1.12 $10^3/\mu\text{L}$ for rats and 3.09 ± 0.59 $10^3/\mu\text{L}$ for euthermic, summer season AGS. Data given as mean \pm S.E.M..

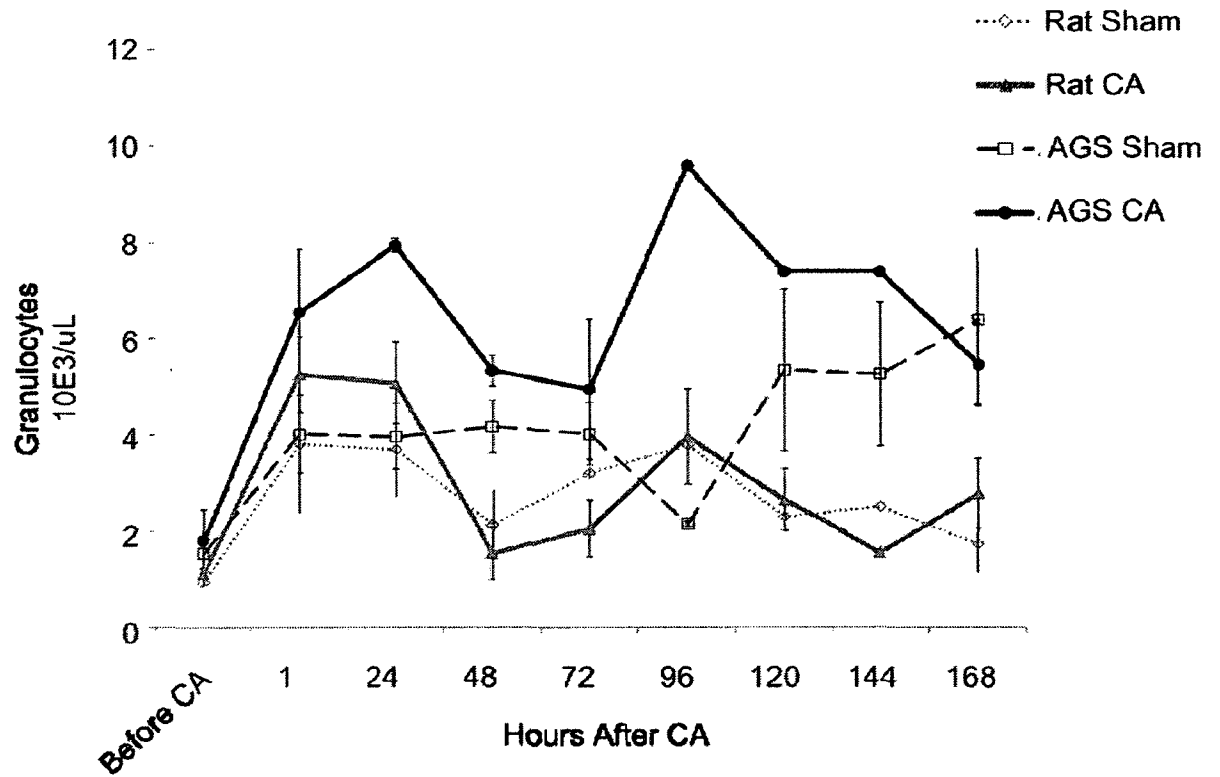


Figure A13. Granulocyte counts during the seven days following treatment. $n=1-6$. Naïve values were $2.30 \pm 0.69 \times 10^3/\mu\text{L}$ for rats and $1.56 \pm 0.34 \times 10^3/\mu\text{L}$ for euthermic, summer season AGS. Data given as mean \pm S.E.M..

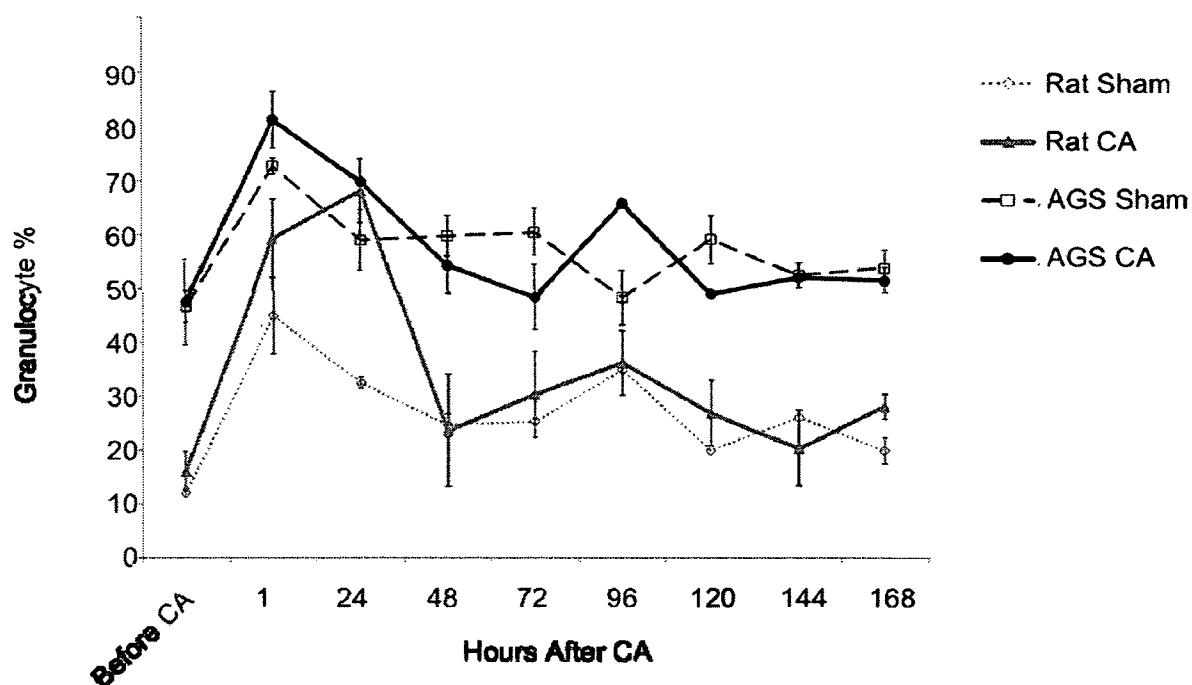


Figure A14. Granulocyte percentage of total white blood cell count during the seven days following treatment. $n=1-6$. Naïve values were $18.00 \pm 3.39\%$ for rats and $49.64 \pm 3.82\%$ for euthermic, summer season AGS. Data given as mean \pm S.E.M..

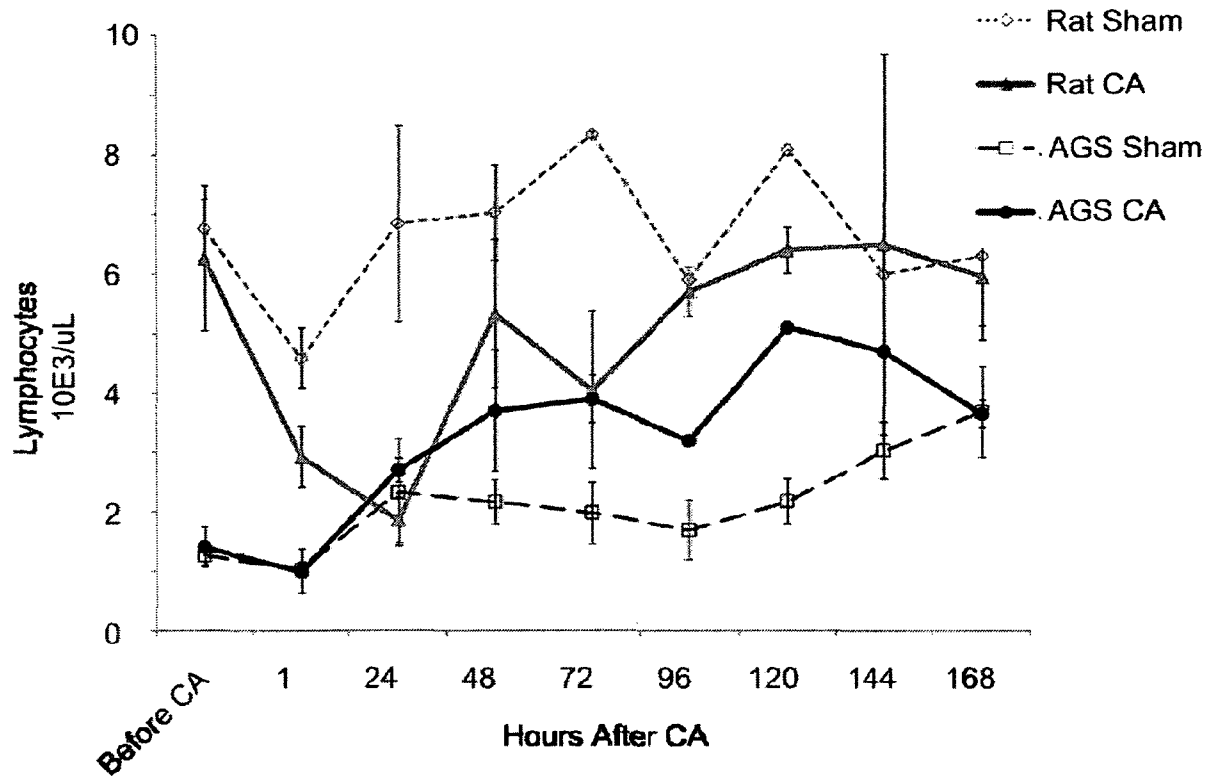


Figure A15. Lymphocyte numbers in rats tended to be the same or higher than AGS before and after CA. $n=1-6$. Naïve values were $8.72 \pm 0.46 \times 10^3/\mu\text{L}$ for rats and $1.16 \pm 0.22 \times 10^3/\mu\text{L}$ for euthermic, summer season AGS. Data given as mean \pm S.E.M..

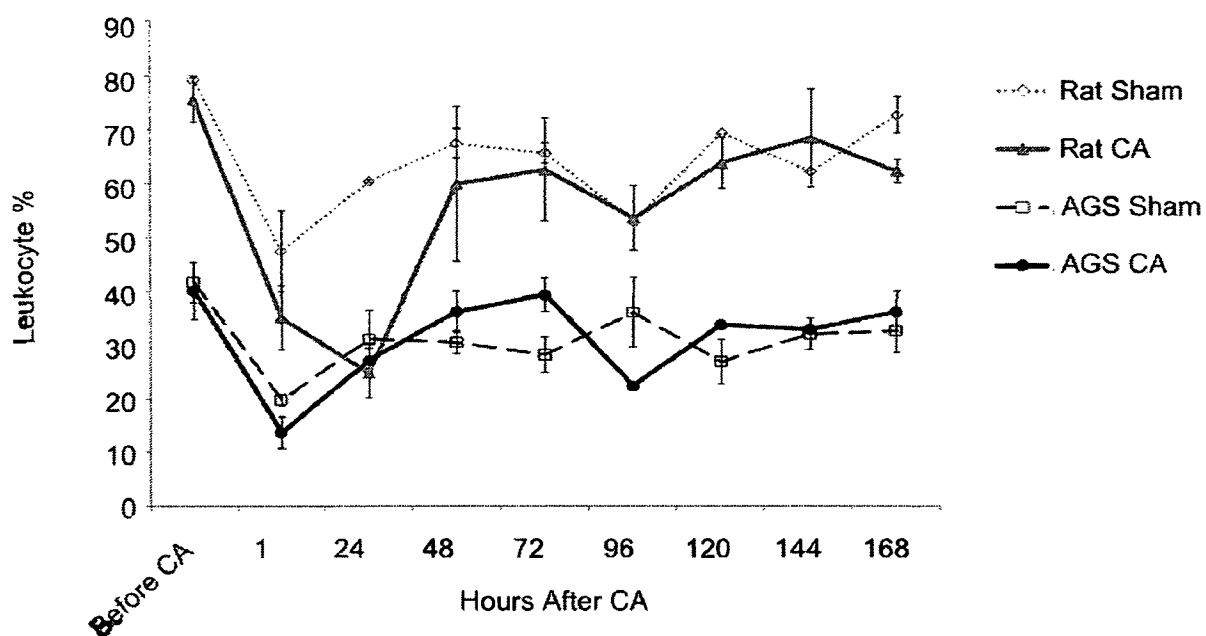


Figure A16. Lymphocyte percentage of total blood cell counts in rats tended to be higher than AGS before and after CA, the exception being at the 24 hour timepoint $n=1-6$. Naïve values were $79.28 \pm 0.60\%$ for rats and $39.74 \pm 3.15\%$ for euthermic, summer season AGS. Data given as mean \pm S.E.M..

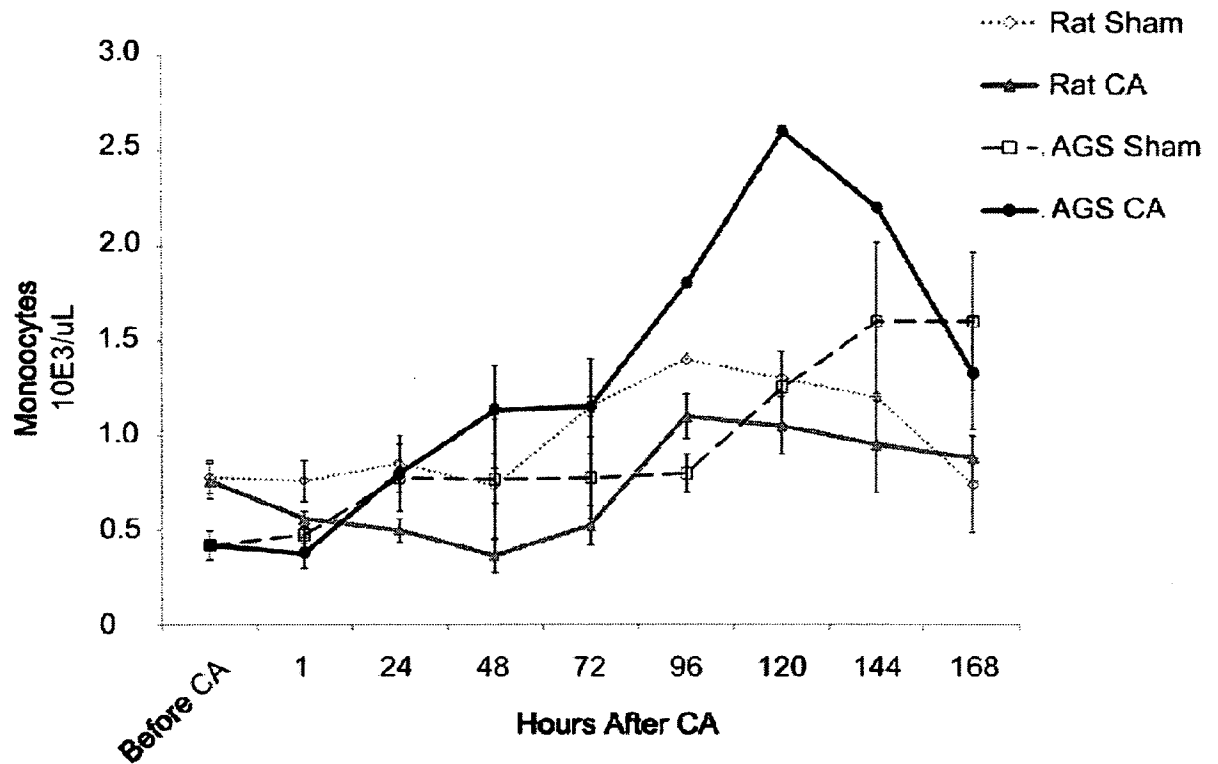


Figure A17. Monocyte numbers were higher in the rats before CA, but seemed to increase more in the AGS after CA, peaking at 120 after I/R. $n=1-6$. Naïve values were 1.10 ± 0.15 $10^3/\mu\text{L}$ for rats and 0.37 ± 0.07 $10^3/\mu\text{L}$ for euthermic, summer season AGS. Data given as mean \pm S.E.M..

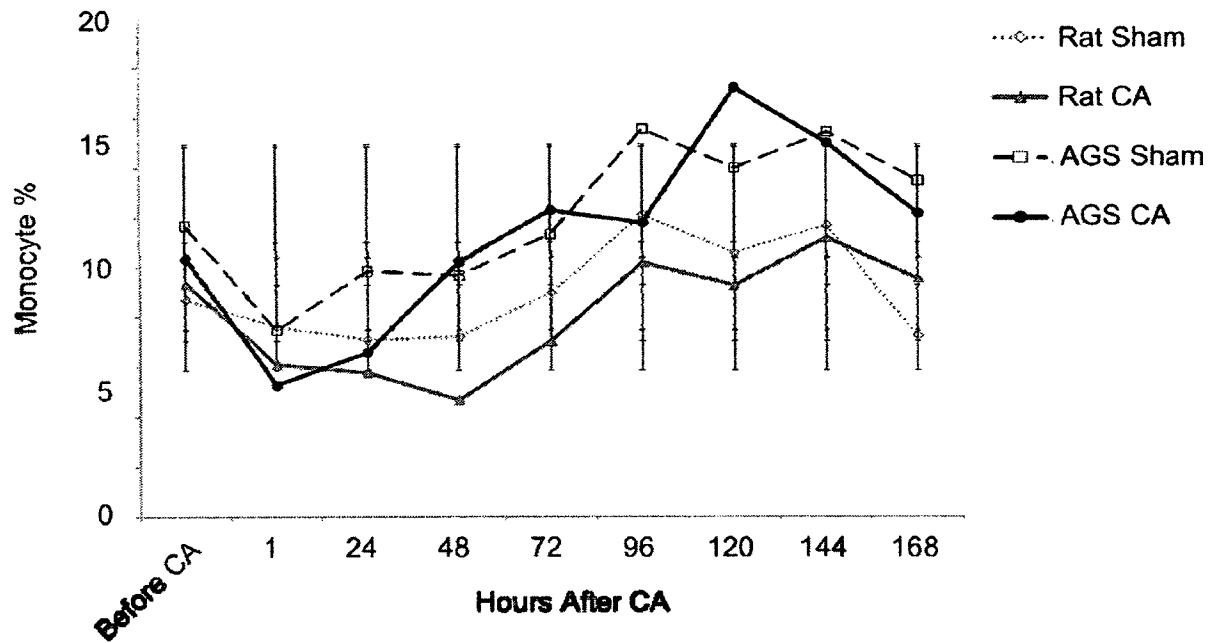


Figure A18. Monocyte percentage of total blood cell counts before and after CA. $n=1-6$. Naïve values were $23.65 \pm 15.26\%$ for rats and $10.61 \pm 1.02\%$ for euthermic, summer season AGS. Data given as mean \pm S.E.M..

Hemorrhagic Shock

The following are data from the HS experiments comparing results from interbout arousal (IBA) AGS, summer euthermic (EU) AGS and the I/R injury positive control, the rat. Animals were subject to hemorrhagic shock (HS) or sham surgery (SHS) where the surgical and experimental procedures were the same, but hemorrhage and reperfusion were not carried out. The graphs below represent data that did not demonstrate a treatment-induced difference between the positive control (rat) and the AGS from either season and/or did not contribute to the overall interpretation of the results. Statistical analysis was not performed.

Blood Chemistries

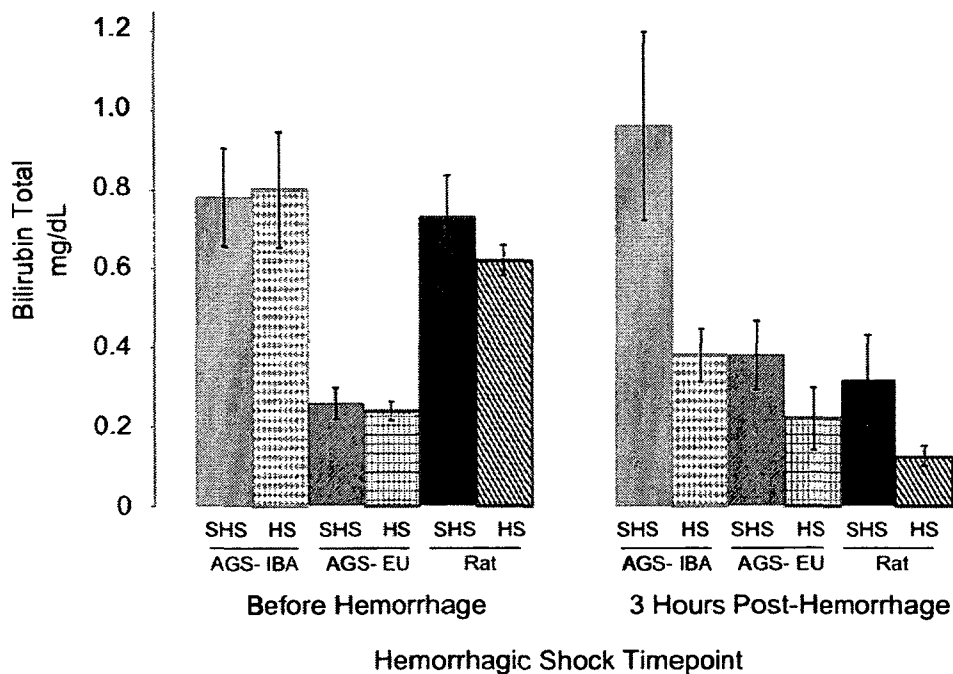


Figure A19. Bilirubin levels did not show a consistent pattern between groups or treatment. $n=5-7$. Naïve values were 0.29 ± 0.07 mg/dL for rats, 0.39 ± 0.07 mg/dL for EU-AGS, and 0.45 ± 0.07 mg/dL for IBA-AGS. Data given as mean \pm S.E.M..

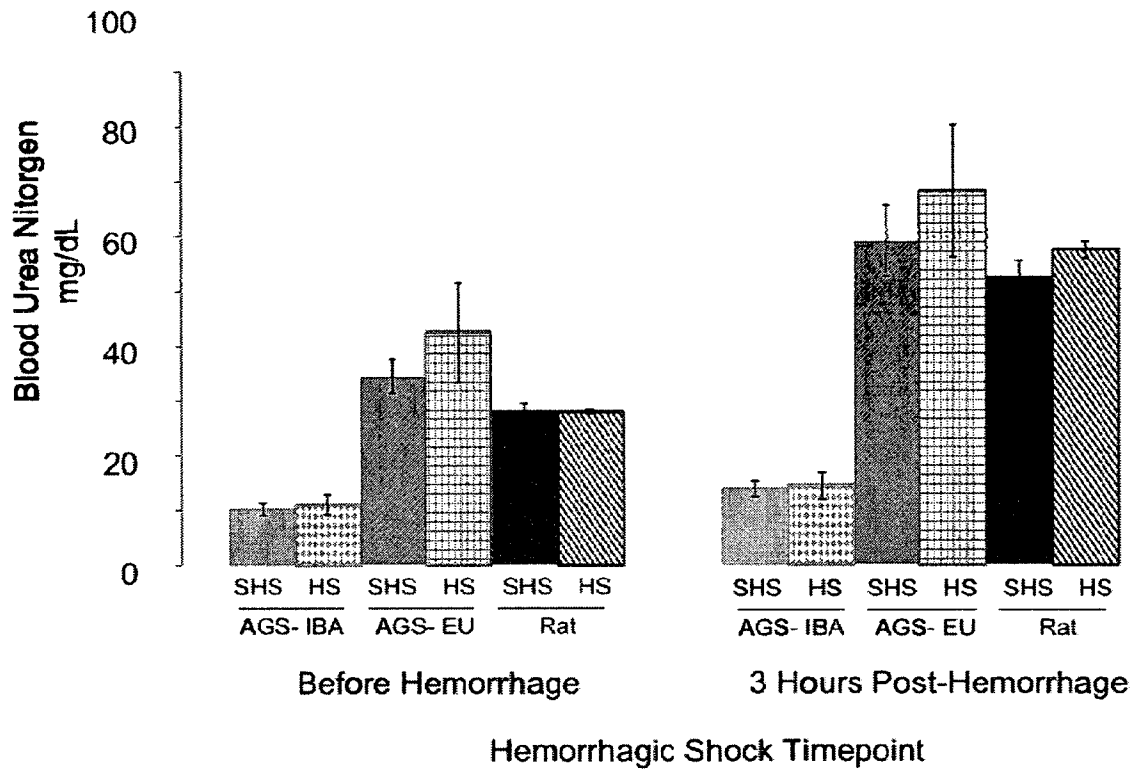


Figure A20. Blood urea nitrogen did not appear to differ between sham and hemorrhaged treatments for any group. $n=5-8$. Naïve values were 19.14 ± 0.86 mg/dL for rats, 27 ± 2.87 mg/dL for EU-AGS, and 9.25 ± 1.22 mg/dL for IBA-AGS. Data given as mean \pm S.E.M..

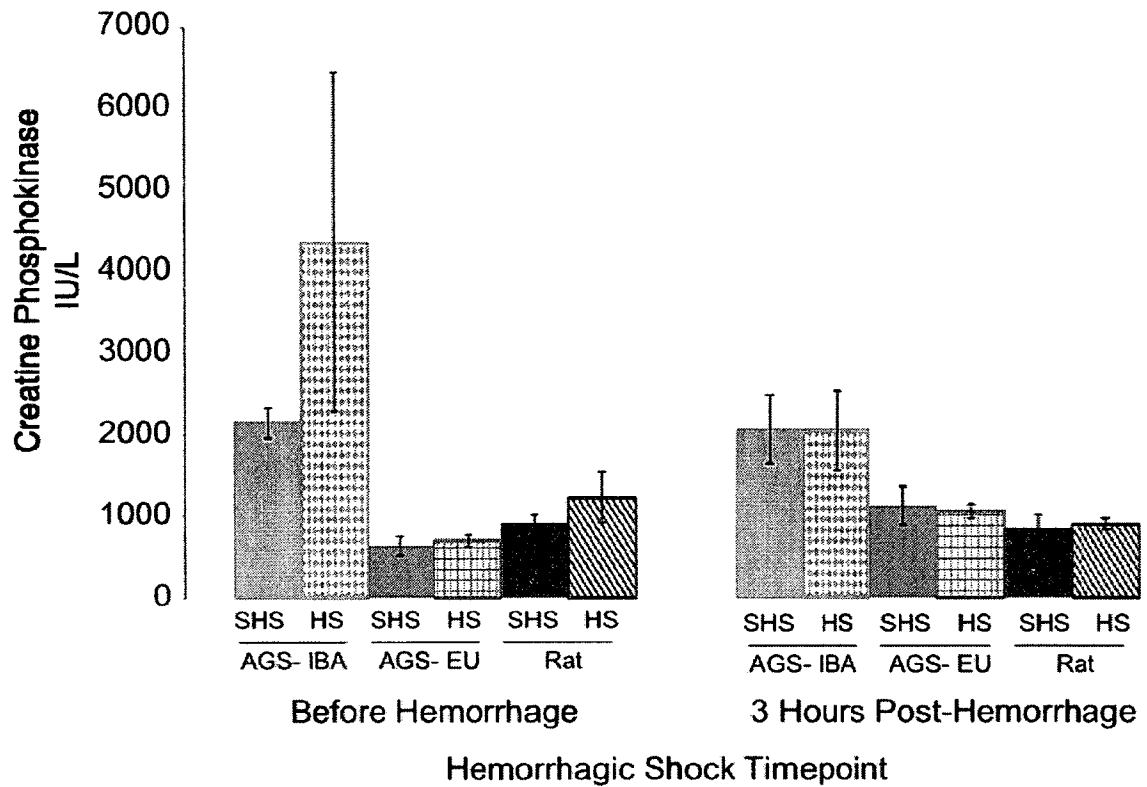


Figure A21. Creatine phosphokinase did not appear to change depending on treatment for any group. $n=5-8$. Naïve values were 1251.67 ± 395.90 IU/L for rats, 613.29 ± 59.64 IU/L for EU-AGS, and 3990.75 ± 1933.99 IU/L for IBA-AGS. Data given as mean \pm S.E.M..

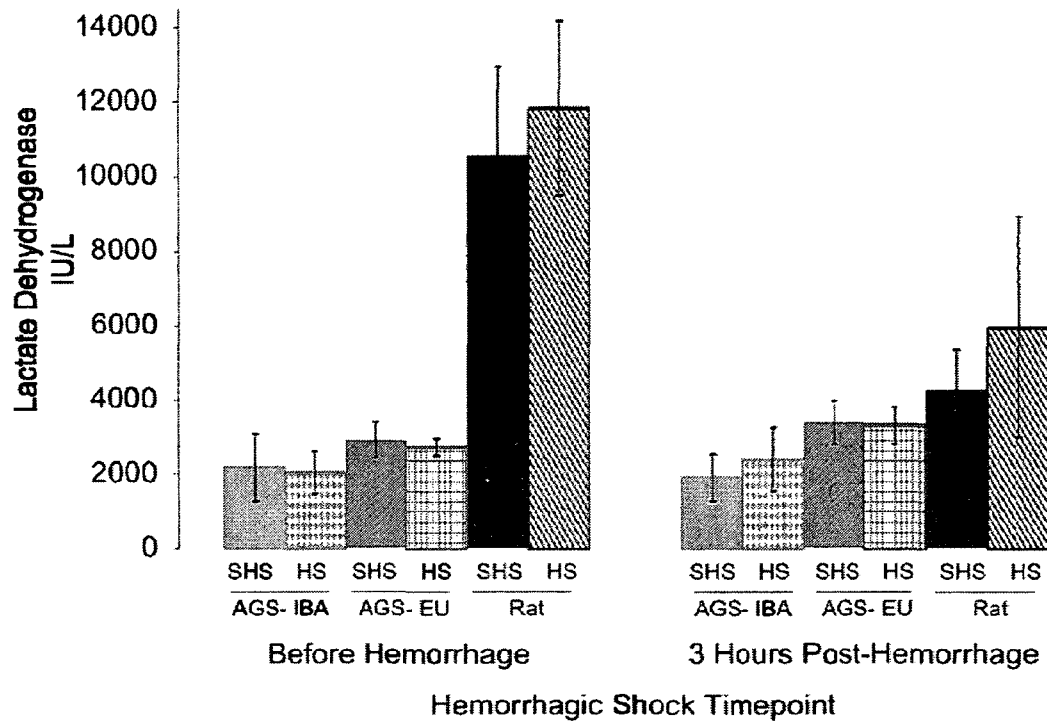


Figure A22. Lactate dehydrogenase tended to decrease in the rats after treatment (sham or hemorrhage) while the other groups appeared unaffected. $n=4-8$. Naïve values were 7553.00 ± 1004.66 IU/L for rats, 3376.57 ± 474.06 IU/L for EU-AGS, and 1816.50 ± 274.36 IU/L for IBA-AGS. Data given as mean \pm S.E.M..

Complete Blood Count

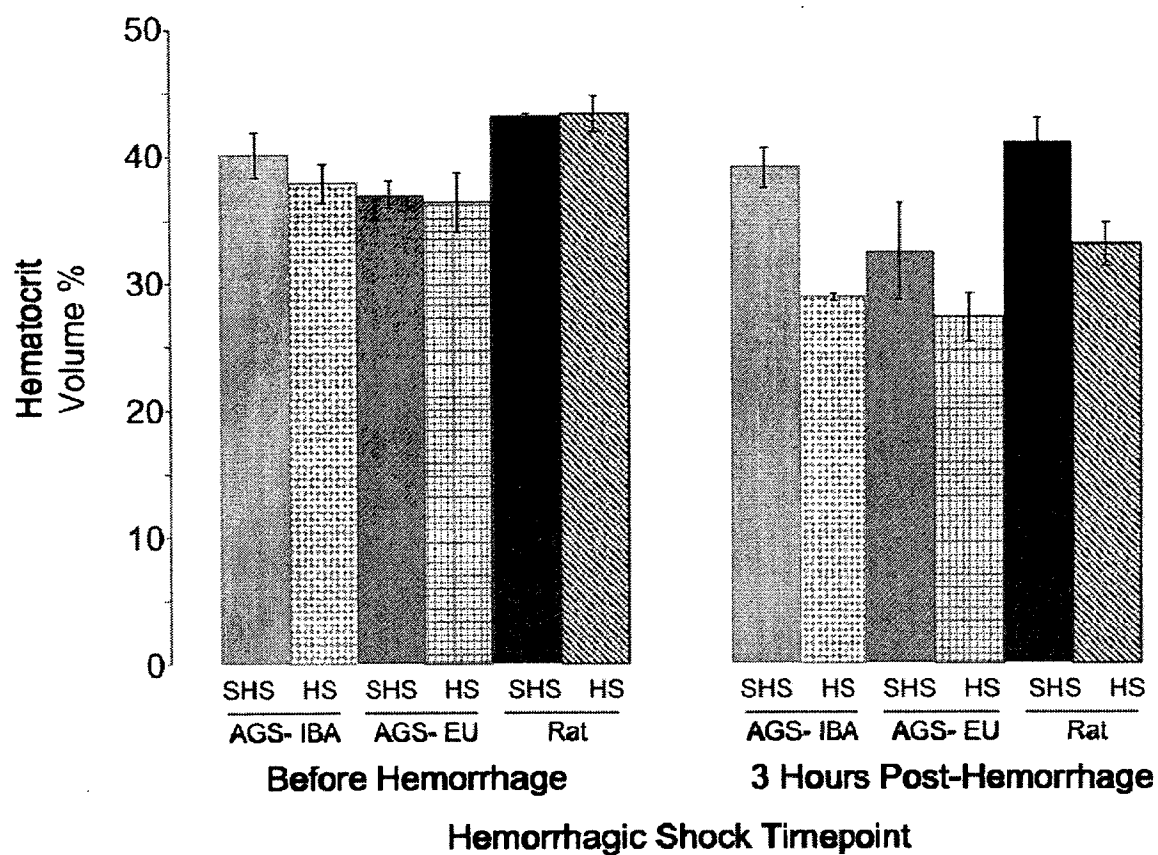


Figure A23. Hematocrit dropped for all hemorrhaged animals. $n=6-8$. Naïve values were 37.41 ± 0.60 Volume % for rats, 36.94 ± 0.92 Volume % for EU-AGS, and 37.45 ± 0.68 Volume % for IBA-AGS. Data given as mean \pm S.E.M..

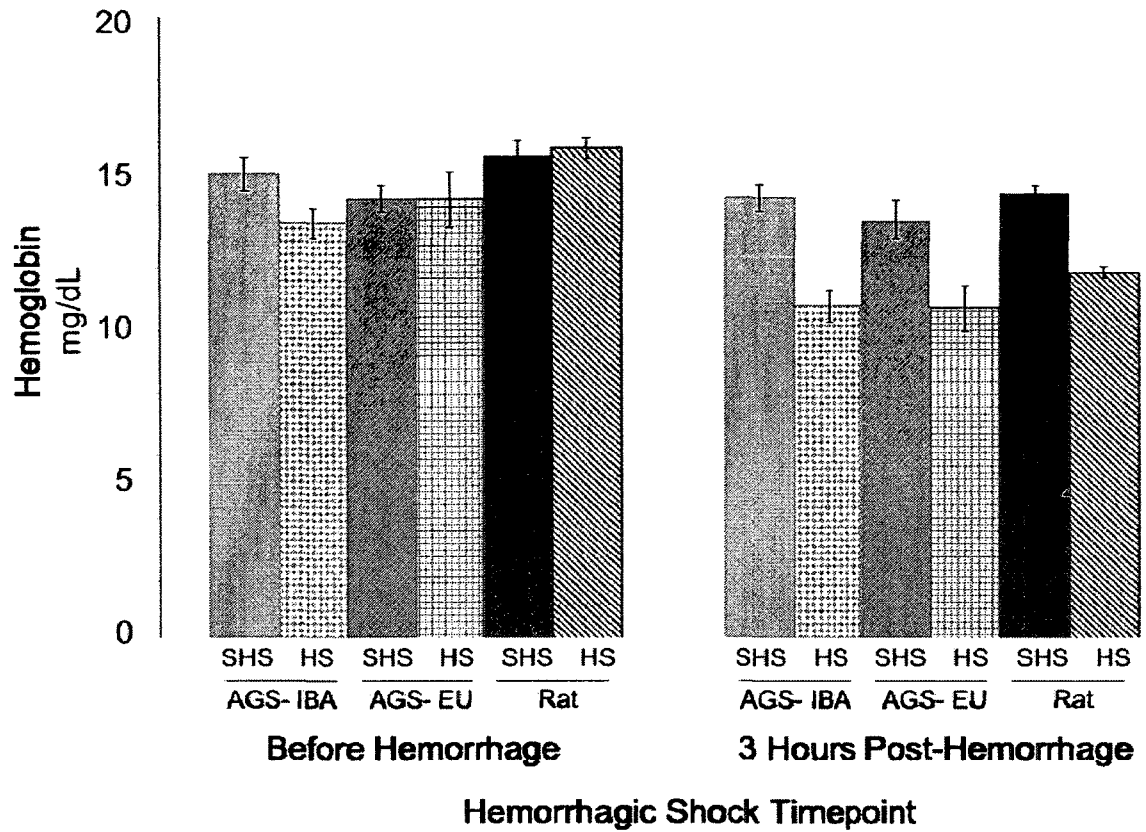


Figure A24. Hemoglobin concentrations dropped for all hemorrhaged animals. $n=6-8$. Naïve values were 14.81 ± 0.23 mg/dL for rats, 14.81 ± 0.51 mg/dL for EU-AGS, and 13.76 ± 0.21 mg/dL for IBA-AGS. Data given as mean \pm S.E.M..

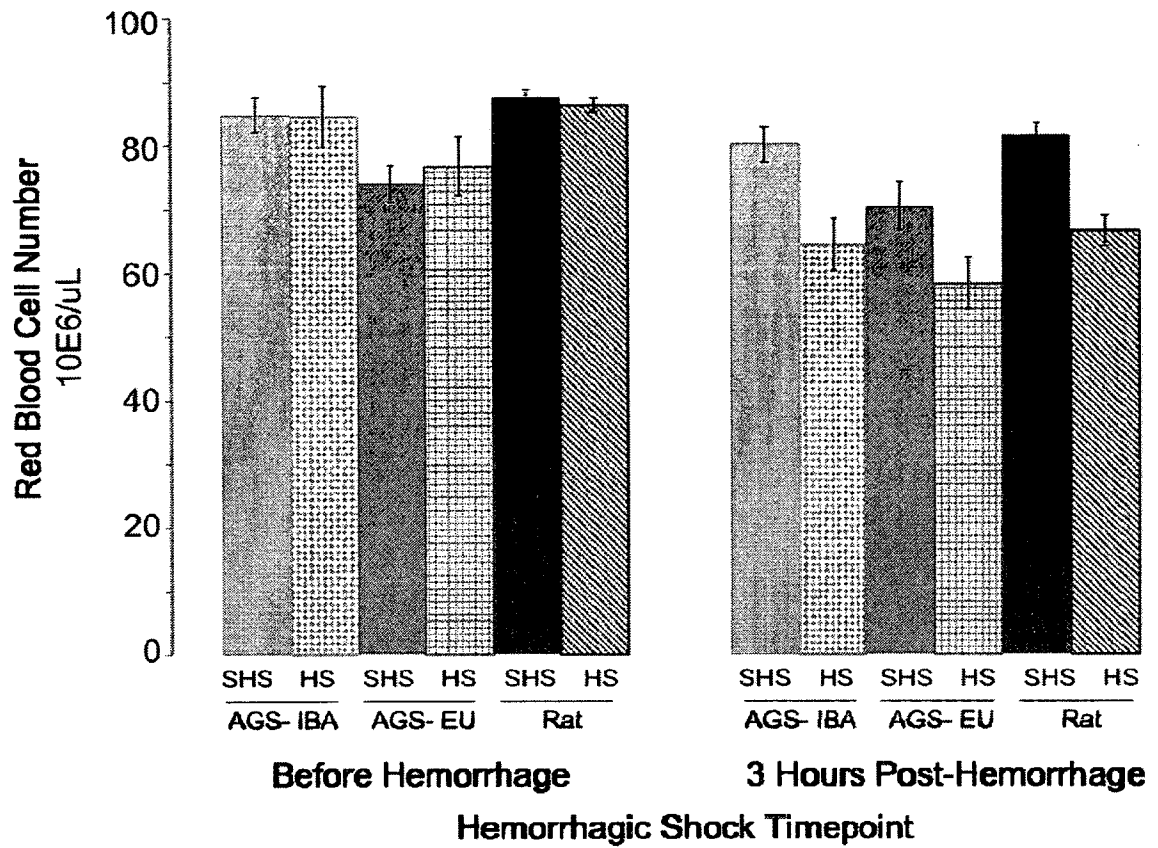


Figure A25. Red blood cell counts went down for all hemorrhaged animals. $n=6-8$. Naïve values were 8.24 ± 0.12 $10^6/\mu\text{L}$ for rats, 7.81 ± 0.11 $10^6/\mu\text{L}$ for EU-AGS, and 7.92 ± 0.24 $10^6/\mu\text{L}$ for IBA-AGS. Data given as mean \pm S.E.M..

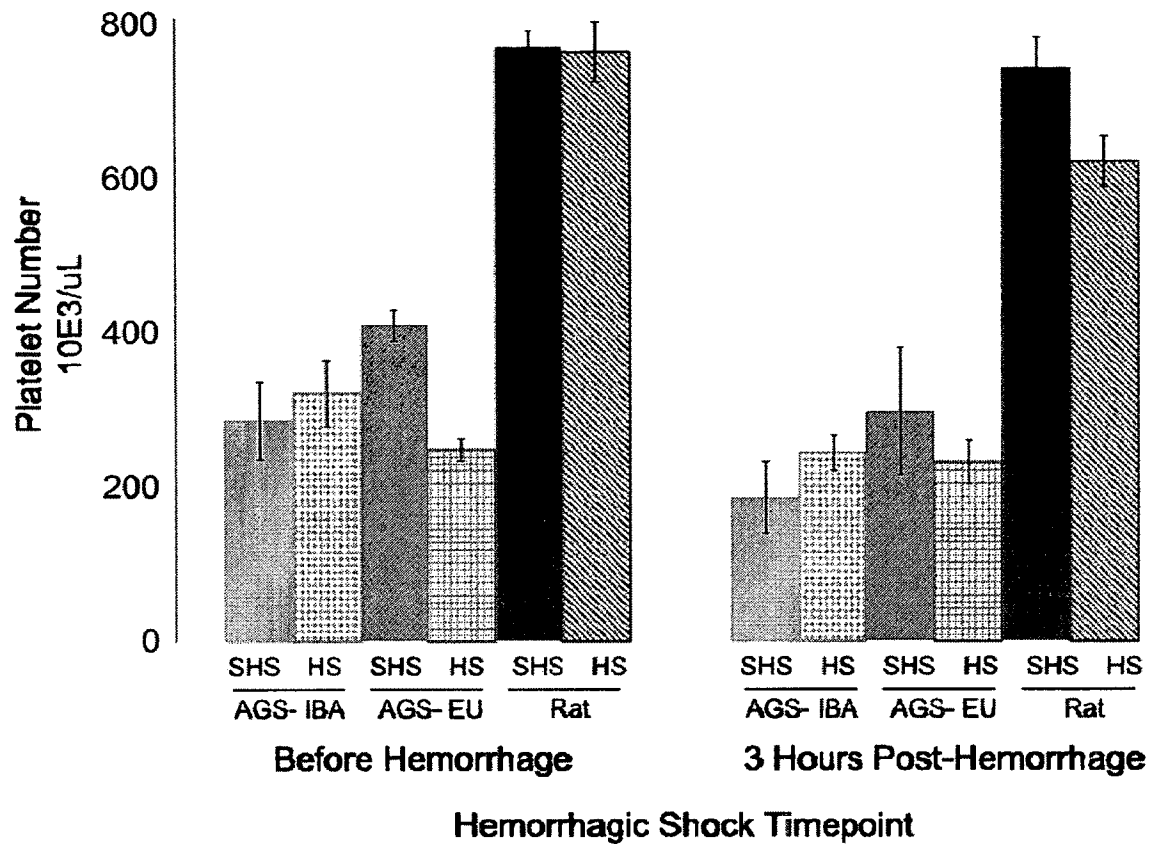


Figure A26. Platelet numbers were higher in the rats regardless of treatment. Treatment did not appear to affect platelet number in AGS, but hemorrhage may have slightly decreased platelet numbers in rats. $n=6-8$. Naïve values were 682.86 ± 82.25 $10E3/uL$ for rats, 266.71 ± 28.14 $10E3/uL$ for EU-AGS, and 238.38 ± 45.42 $10E3/uL$ for IBA-AGS. Data given as mean \pm S.E.M..

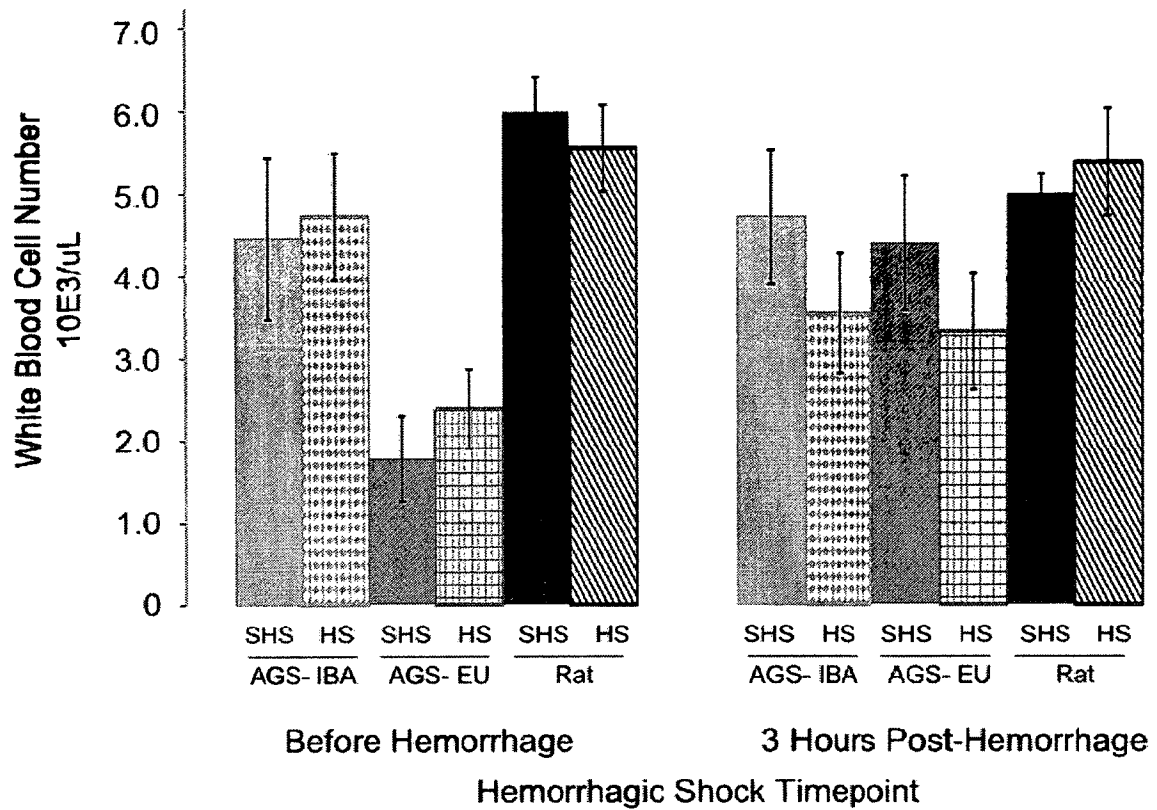


Figure A27. White blood cell counts were unaffected by treatment; however, EU-AGS initial had lower counts that rose after treatment to resemble levels of the other groups. $n=6-8$. Naïve values were 5.77 ± 0.73 $10E3/uL$ for rats, 3.09 ± 0.59 $10E3/uL$ for EU-AGS, and 4.86 ± 0.52 $10E3/uL$ for IBA-AGS. Data given as mean \pm S.E.M..

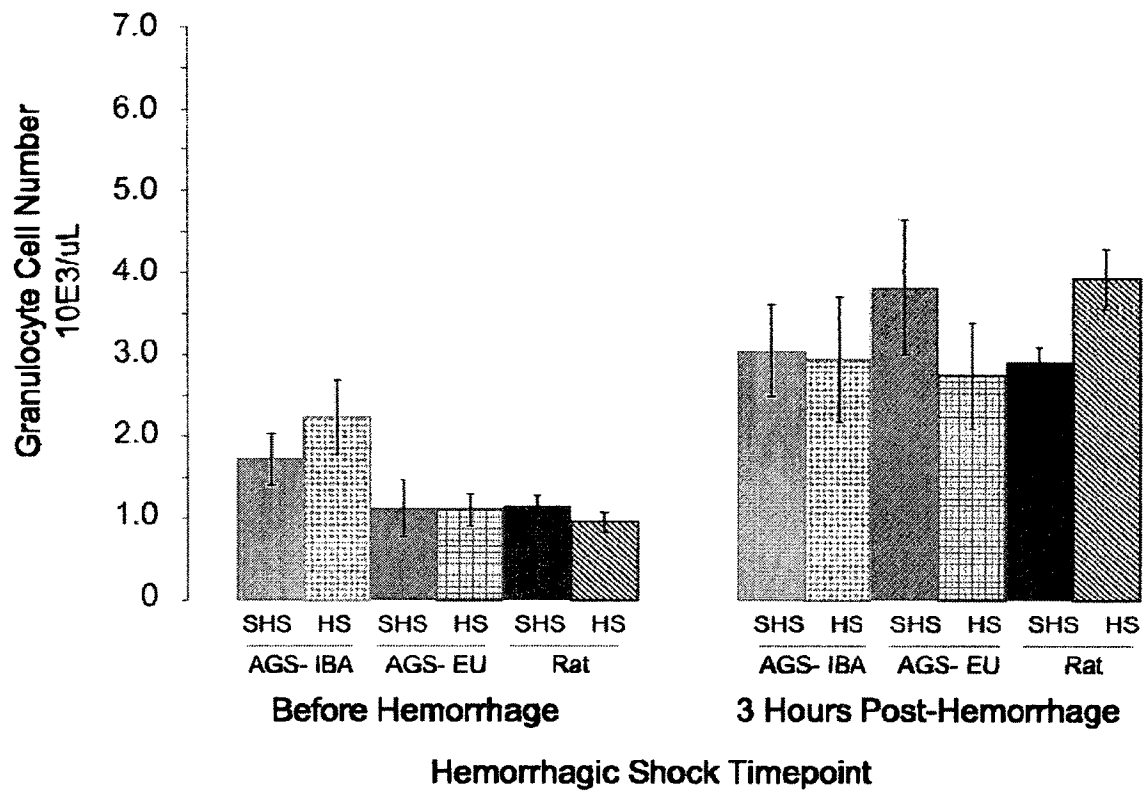


Figure A28. Granulocyte numbers increased during the protocol for all groups. Rats tended to have a greater increase in the hemorrhaged group versus the sham animals. $n=6-8$. Naïve values were 0.91 ± 0.13 $10^3/\mu\text{L}$ for rats, 1.56 ± 0.34 $10^3/\mu\text{L}$ for EU-AGS, and 1.89 ± 0.29 $10^3/\mu\text{L}$ for IBA-AGS. Data given as mean \pm S.E.M..

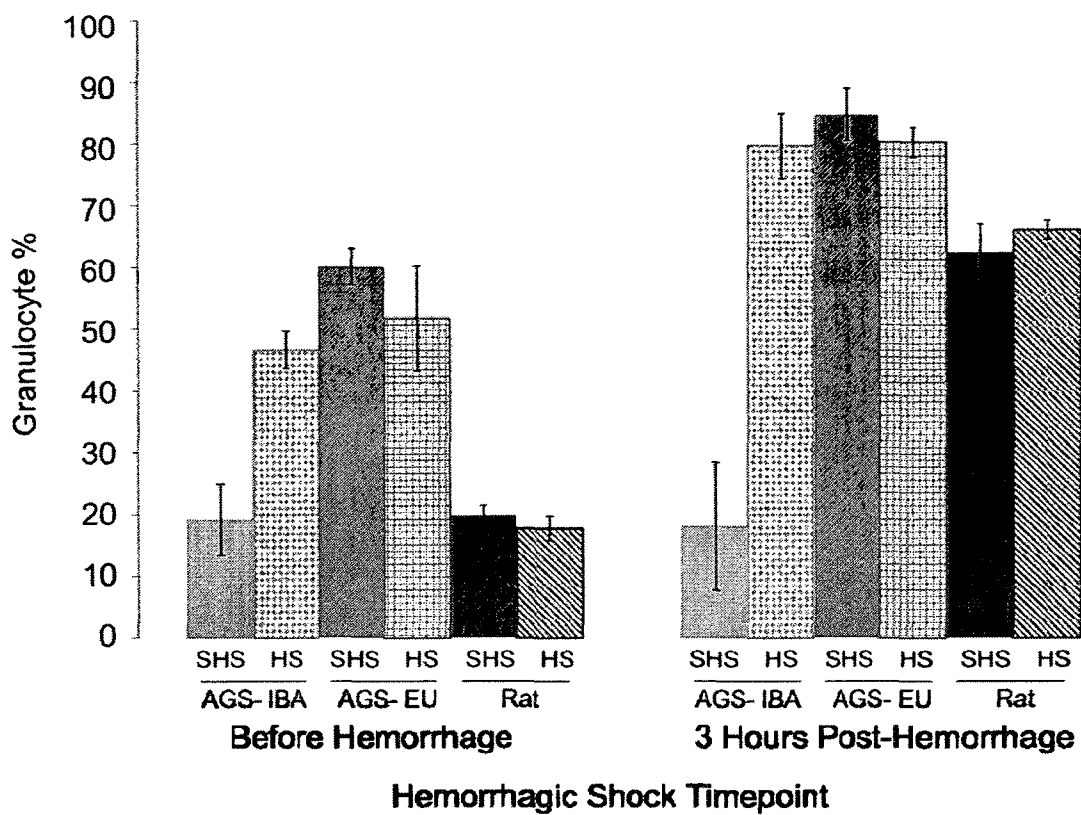


Figure A29. Granulocyte percentage of the white blood cell population increased during treatment for all but the IBA-AGS sham animals. In other groups, the increase did not appear to depend upon treatment. $n=6-8$. Naïve values were $16.17 \pm 0.94\%$ for rats, $49.64 \pm 3.82\%$ for EU-AGS, and $38.40 \pm 1.93\%$ for IBA-AGS. Data given as mean \pm S.E.M..

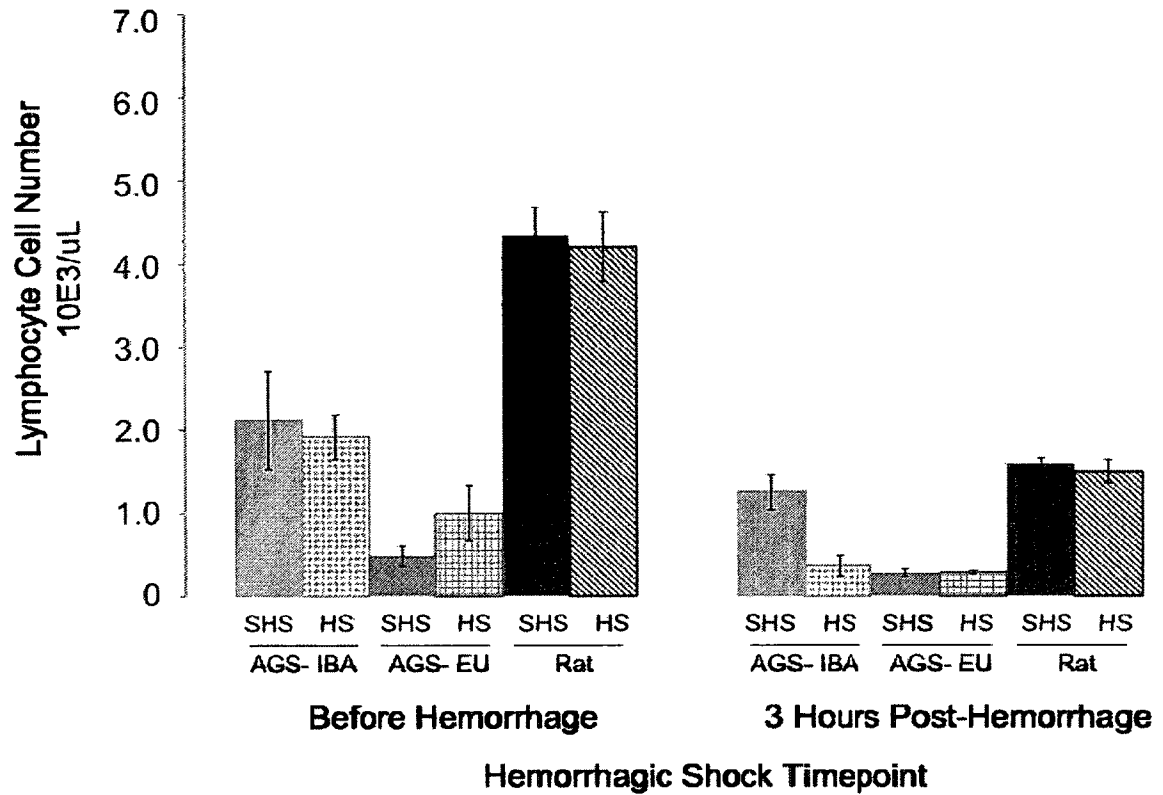


Figure A30. Lymphocyte numbers decreased during the protocol for all groups. Rats tended to have a greater decrease than either of the AGS groups, regardless of treatment. Hemorrhaged IBA-AGS tended to decrease more in the hemorrhaged versus sham animals. $n=6-8$. Naïve values were 4.49 ± 0.59 $10^3/\mu\text{L}$ for rats, 1.16 ± 0.22 $10^3/\mu\text{L}$ for EU-AGS, and 2.30 ± 0.20 $10^3/\mu\text{L}$ for IBA-AGS. Data given as mean \pm S.E.M..

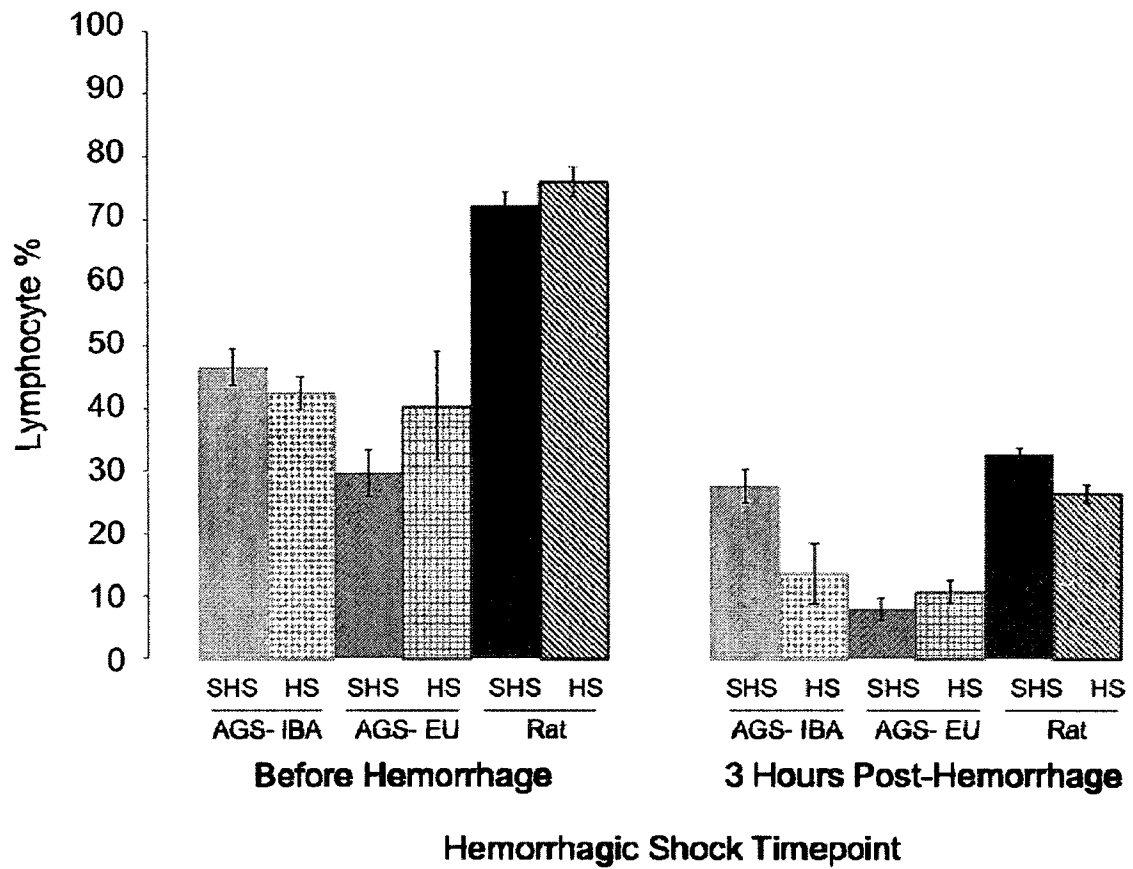


Figure A31. Lymphocyte percentage decrease for all groups with the rats and IBA-AGS appearing to show a greater decrease in the hemorrhaged group over the sham surgeries. $n=6-8$. Naïve values were $77.07 \pm 1.44\%$ for rats, $39.74 \pm 3.15\%$ for EU-AGS, and $48.58 \pm 2.29\%$ for IBA-AGS. Data given as mean \pm S.E.M..

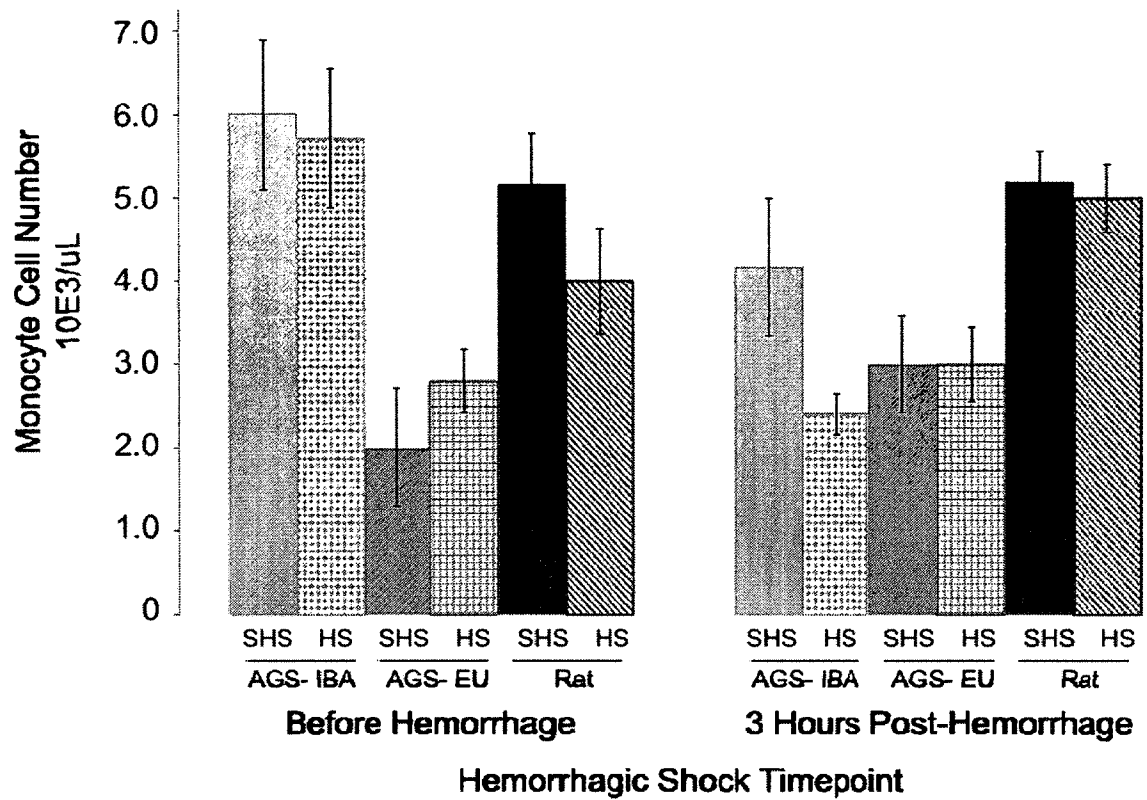


Figure A32. Monocyte numbers looked to have decreased during the protocol for all The IBA-AGS with the hemorrhaged animals appearing to have a greater decrease than the sham. Numbers seemed to remain unchanged for the other groups and treatments. $n=6-8$. Naïve values were 2.63 ± 0.86 $10E3/uL$ for rats, 0.37 ± 0.07 $10E3/uL$ for EU-AGS, and 0.68 ± 0.08 $10E3/uL$ for IBA-AGS. Data given as mean \pm S.E.M..

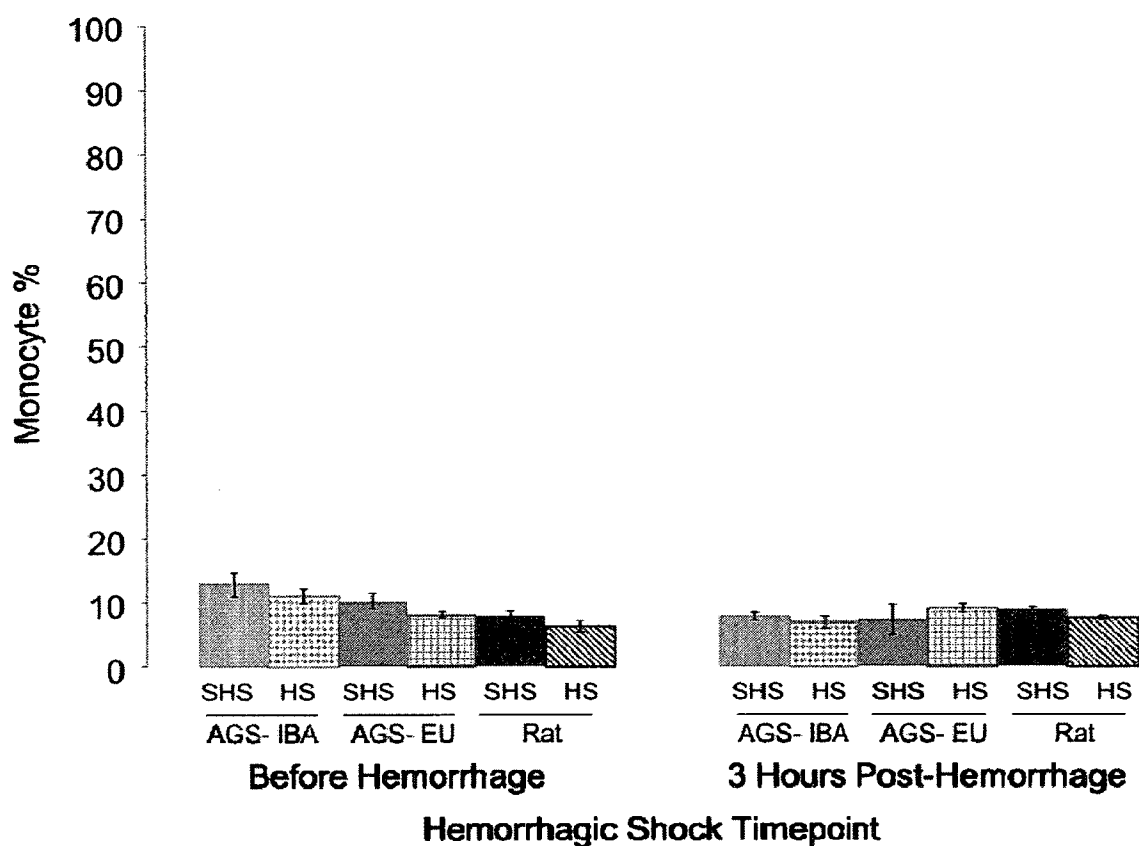


Figure A33. Monocyte percentage gave the impression of decreasing for the IBA-AGS animals, both sham and hemorrhaged. All groups maintained a low monocyte percentage before and after treatment. $n=6-8$. Naïve values were $6.04 \pm 0.48\%$ for rats, $10.61 \pm 1.02\%$ for EU-AGS, and $13.03 \pm 0.79\%$ for IBA-AGS. Data given as mean \pm S.E.M..

Cytokine Analysis

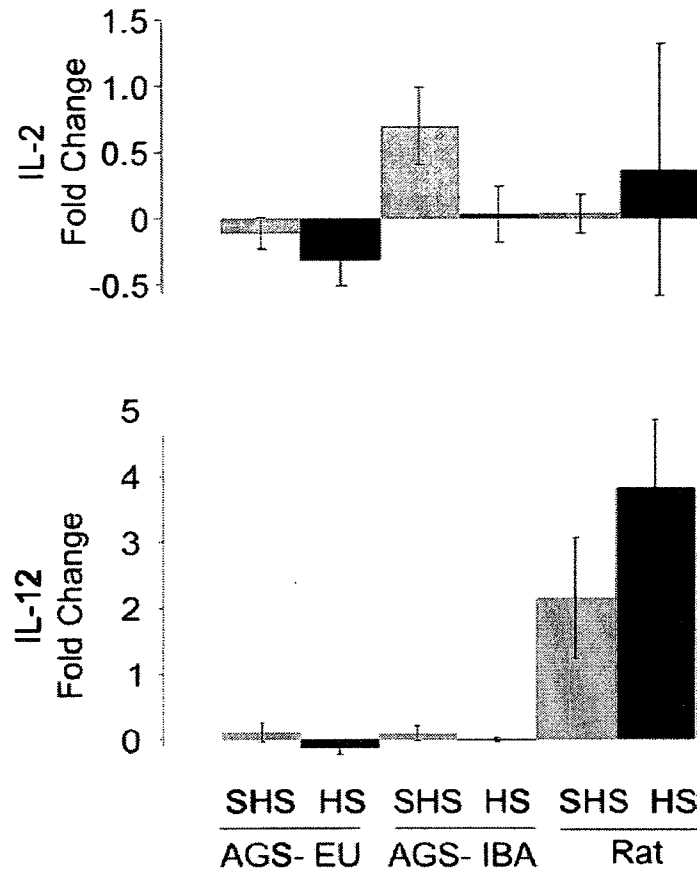


Figure A34. Cytokine levels for IL-2 did not show I/R based increases in the I/R injury prone rat. IL-12 levels appeared to be greater in the rat compared to AGS (either season) regardless of treatment. Differences in IL-12 levels may be due to antibody affinity differences between the two species. IL-12 showed a magnitude or order difference in the naive rats and IBA- or EU-AGS. $n=6-8$. For IL-2 naïve values were 827.64 ± 177.12 pg/mL for rats, 549.33 ± 311.95 pg/mL for EU-AGS, and 180.35 ± 35.27 pg/mL for IBA-AGS. For IL-12 naïve values were 1385.84 ± 256.83 pg/mL for rats, 37.26 ± 1.48 pg/mL for EU-AGS, and 29.22 ± 5.09 pg/mL for IBA-AGS. Data given as mean \pm S.E.M..

¹H-NMR Metabolomics

The following spectrum is from the ¹H-NMR metabolomics experiments run on the plasma samples from the HS experiments. The two dimensional COReLation SpectroscopY (COSY) spectrum was used to verify peak identity for the quantitative metabolomic analysis. Statistical analysis was not performed.

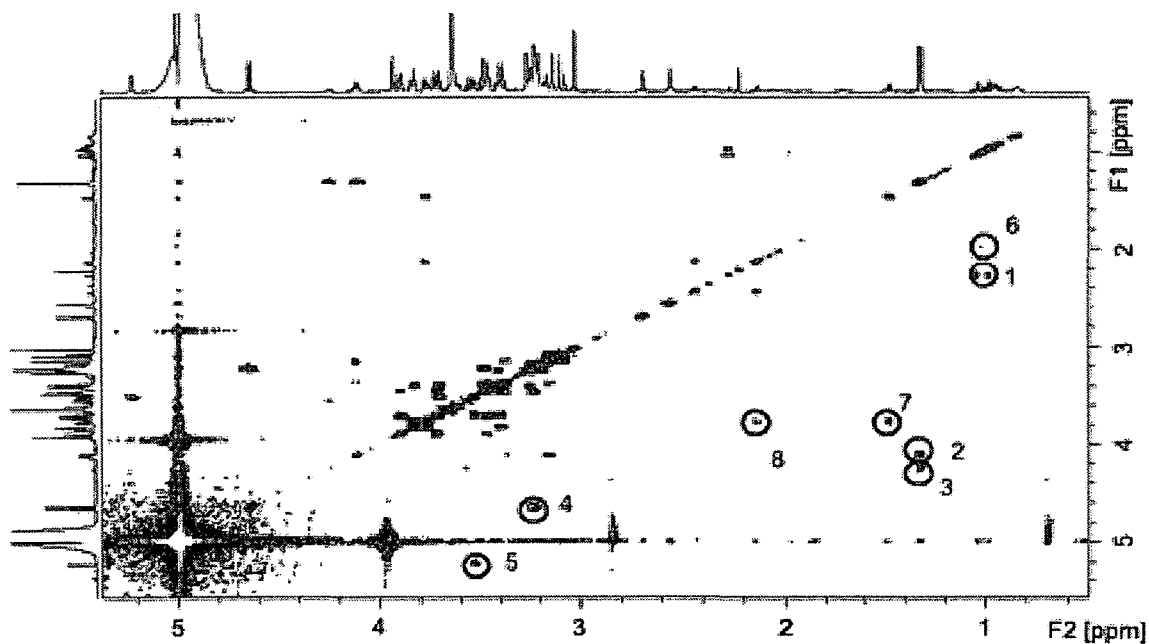


Figure A35. Two dimensional COSY ¹H-NMR spectra of AGS total plasma. NMR peak assignments: 1, valine; 2, lactate; 3, threonine; 4, α-glucose; 5, β-glucose; 6, isoleucine; 7, alanine; 8, glutamine.

Appendix B. Surgical Standard Operating Procedures (SSOPs)

Cardiac Arrest SSOP

Preparation of Surgical Site

Shave throat (if tracheotomy is planned), right and left inguinal areas, dorsal surface of both forelegs and the hind leg not involved in femoral vessel cannulation. Scrub incision sites using 70% alcohol and 100% betadine solution beginning with betadine and alternating with 70% isopropyl alcohol for three times (three swabs betadine and two swabs 70% alcohol), ending with 100% betadine. Use circular, inside-to-outside motions when scrubbing incision site so as not to contaminate the innermost region.

Surgical Procedure

1. Open pressure gauge pack and set up pressure transducer.
2. Prepare injectables (see pg. 10) and fill two cannulae with hep-saline with 1cc syringe and 23 ga. leur stub adapter (Becton Dickinson; reorder # 427565) for PE-50; 26ga leur stub for Tygon. Fill one 10cc syringe with sterile saline. Leave sterile ends in pack.
3. Anesthetize with 5% isoflurane and a 30:70 mixture of O₂ (400-500mL/min) and N₂O (1L/min) via scavenger fluovac mask. This insures stable physiological levels for P_{O2}, P_{CO2} and pH.
4. Begin endotracheal intubation: Open sterile intubation pack. For AGS, surgical tape (for example, a 1cm wide strip of 3M Micropore™) is wrapped around the endotracheal catheter approximately 1in from tip to increase the outer diameter of the catheter by 1-2mm and make a tighter fit within the trachea. Make sure animal is lying face up on intubation rodent stand and take animal off anesthesia mask and be sure that animal is secured by tooth strap and (optional) sliding bars located on stand itself. Next, insert otoscope (with speculum attached and light turned on) (otoscope and intubation setup found at www.hallowell.com/index.php?pr=Product_Catalogue) against the palate of the mouth (with the

curved slide facing downward) while holding and deflecting the tongue to the side. Next, place a drop of lidocaine gel (Lidocaine Hydrochloride Jelly USP, 2% Rx; 30 mL, NDC 17478-711-30 or equivalent) to the tip of the lidocaine applicator and apply it the epiglottis (arch shaped and located just above the vocal cords) while still holding the otoscope and speculum against the palate of the animal's mouth with the opposite hand. Lidocaine gel application should reduce epiglottis spasm as well as irritation to this sensitive region. Put animal back on anesthesia mask (rats 2.5% isoflurane; AGS 3-3.5% isoflurane) for two minutes.

5. Take animal off mask and reinsert otoscope/speculum unit as described earlier. This time use the endotracheal catheter (14ga 1.75in; BD Insyte Autoguard, shielded iv catheter, ref# 381467 guided with a bent, custom blunt biomedical 17gax4in needle (17TWx3, Popper & Sons, New Hyden Park, NY #7427) or flexible metal stilette attached to a syringe "handle" and try to insert it (with curved side facing up) into the trachea located via the opening between the two vocal cords. Movement of the vocal cords should be slowed down enough in order to maneuver this technique. The catheter is suitably within the trachea when the orange hub is just outside the incisors; only the orange hub is outside the oral cavity. In AGS the orange hub should remain within the oral cavity and not extend any farther forward than the back side of the incisors. Immediately check if the catheter is truly in the trachea and not in the esophagus by holding a stainless steel instrument in front of the open end of the catheter (orange hub) so that condensation of respiratory moisture can be seen. If no moisture is visible, then it means that the catheter is in the esophagus.

If the first intubation attempt is unsuccessful, repeat the aforementioned steps.

Discontinue surgery if more than two tries are needed to execute intubation. Return animal to home cage for at least one week before repeat attempt.

Suture catheter to lip to avoid dislodging catheter during resuscitation. Close with 3-0 Prolene, simple interrupted stitch, at least three throws.

For terminal, non-recovery procedures a tracheotomy may be substituted for oral intubation. The trachea will be exposed as described above. A small hole will be made between the cartilaginous rings in the trachea and the 14ga 1.75in; BD Insyte catheter will be inserted between the tracheal rings. The catheter will be secured in the trachea with 1 or 2 sutures using 3-0 silk.

6. Ventilate. If needed to maintain SO_2 within the normal range for the species, apply 1-5cm PEEP by immersing expiratory tubing into a 1-5cm high column of water

7. Shave throat (if tracheotomy is planned), right groin, and dorsal surface of remaining legs (for ECG electrodes); scrub shaved areas using 70% alcohol and 100% betadine solution beginning with betadine and alternating with 70% isopropyl alcohol for three times (three swabs betadine and two swabs 70% alcohol), ending with 100% betadine. Use circular, inside-to-outside motions when scrubbing incision so as not to contaminate the innermost region.

8. Record vitals: Cover eyes with sterile ophthalmic ointment for lubrication (e.g. vetropolycin, neo-poly-bac, etc.). Lubricate rectal thermometer with K-Y jelly and insert 8-10cm into rectum. Locate the temporalis muscle by palpating the small bulge between dorsal midline of the skull and the caudo-dorsal aspect of the eye, and insert the temporalis thermocouple needle 2-4mm into the muscle with the needle pointing rostrally (toward the nose). Insert EKG leads subcutaneously (ground to hind leg; negative to left front leg, positive to right front leg) and secure both EKG leads and thermocouples/needles with surgical tape. Monitor SO_2 with pulse-oximeter if applicable. Record values on surgery log and anesthesia record.

9. Don sterile gloves, drape and open pack and arrange surgical instruments.

- Small vessel scissors (protect with tip of yellow pipette)
- 45o 5/45 sharp forceps (protect with tip of yellow pipette)

- Blunt; bent 16ga/2.5" needle (may bend, blunt and file needle from IV catheter used for endotracheal catheter) or purchase from Popper&Sons, 17TWx3 custom blunt popper biomedical needle (cat# 7427, <http://popperandsons.com/index.asp>)
- Fine, straight rat-toothed forceps (Miltex 6-106; ~1mm or 1/16" width x 5 inches long)
- 90° curved hemostats (5.5 inch long; Roboz RS 7291)
- 2 x 8 inch smooth inner surface curved (~90°) forceps (for teasing apart and holding vessels; Paragon #4004-96 <http://www.paragonmedical.com/>)
- Curved, blunt scissors for cutting skin and separating skin and muscle (Roboz; RS-6891)
- 4 inch curved forceps for holding cannula
- 2 ½" forceps (rough)
- Needle holder
- Sterile drape with hole for throat and groin.
- (Optional) Y-Leur adapter; used to connect both epinephrine and NaCHO₃- to venous cannula line for immediate injection during resuscitation.
- Group of 6 inch cotton tipped applicators (quantity based on surgeon's preference)
- Small scissors for cutting knots
- ½" pile of gauze wrapped in tin foil (Flush with saline, clean out blood clots, cover with saline wetted gauze)

- (Optional) Extra, 4 x 23ga blunt connectors, 6 x 23ga. Leur stub adapters (for PE-50) 6x 26 ga leur stub adapters (for Tygon tubing)
- 1 silk needle w/suture (for securing endotracheal catheter to lip after intubation)
- 4-7 x 15 inch pieces of silk thread necessary for anchoring and tying off vessels during cannulation
- 2-3 x 3-0 Prolene sutures for skin closure (3/8 19 mm; FS-2, 18" [45 cm] Non-absorbable)
- (Optional) Retractor for throat if doing tracheotomy
- Follow procedure on Excel data sheet

*Note If tracheotomy is planned:

- IV catheter (same for rats and AGS; BD/Insyte; H3124A; 14ga x 1.75 inch; 2.1 x 45mm)
- 3-0 Prolene (when closing)

10. Cannulate femoral vein and then artery, flush with saline and close temporarily with sutures. Polyethylene catheters (Tygon tubing; 0.375-mm ID, 0.75-mm OD; Norton, Akron, OH or PE-50 catheter for vein and artery), will be introduced ~ 2 cm into the right femoral artery and vein for acute blood pressure recording, blood sampling, and drug infusion; or 6 cm for chronic cannula placement. Mean arterial blood pressure (MABP) will be measured via an indwelling femoral arterial catheter connected to a precalibrated Statham pressure transducer and will be recorded continuously. Arterial blood gases and pH, and plasma glucose, will be measured in microsamples (75-100uL). (Total volume not to exceed 1.5mL.) If cannulation of the right femoral artery or vein is unsuccessful, cannulation will be completed on the left femoral artery

and/or vein following the same procedure described above in "Preparation of surgery site" and "Incision site".

11. Paralyze: Inject 0.3mL Vecuronium (1mg/mL) iv. Watch to see that animal stops breathing. Connect ventilator (humidified with mucomist (1.4mL/70mL H₂O); pressure dampened with rebreathe bag at, approximately 53 rpm, SV 2.0mL). Decrease isoflurane to 0.5%.

12. Sample and adjust blood gases; adjust stroke volume, SV, (typically between 2 – 3 mL) and %O₂ (typically between 450 and 500mL/min) to adjust blood gases (AGS P_{CO2}: 30-84mm Hg; P_{O2} 41-73 mm Hg [P_{CO2} of 60mm Hg and P_{O2} of 60mm Hg is typical for AGS]; Rat P_{CO2}: 35-40; P_{O2} 100-110 [110-130 is acceptable].

13. Monitor T_{rec} and T_{temporalis} until between 36.5 and 37.5°C.

14. At least 10 min after previous injection of vecuronium, inject vecuronium (0.3mL of 1mg/mL, i.v.) and FLUSH immediately with 0.3mL hep-saline (6.7 IU/mL), two minutes later disconnect ventilator. OBSERVE THAT ANIMAL IS NOT BREATHING. If animal continues to breathe (>2 breaths) inject another 0.3mL of vecuronium, wait 10min, and repeat this step. If animal is not breathing prepare to resuscitate. (Connect two-way leur adapter primed with (10 ug/mL) and NaHCO₃ (8.4%) for immediately delivery; change BP scale to 0-50; turn off isoflurane vaporizer; turn N₂O flow meter to 0 and increase O₂ to 2L/min and respiratory rate to 80 bpm. NOTE last vecuronium injection and start of CA or sham procedure and Resuscitation in LabScribe.

15. Resuscitate by reconnecting ventilator. (Positive end-expiratory pressure, PEEP, may be used as needed to increase functional residual capacity of the lungs and improve gas exchange. For PEEP, the end of the expiratory tube is placed under 1-5 cm of water.) Immediately inject 1mL/kg fresh epinephrine (10ug/kg , and apply rapid manual chest compressions at a rate of 200/min to base of the heart until BP is at least 50 mm Hg and is maintained by spontaneously beating heart for ≥ 10 sec. If not resuscitated after 1 min, give a second epinephrine dose. Once

HR is spontaneous and MABP > 50 mm Hg, immediately inject 0.9 cc NaHCO₃. Resuscitation is discontinued after 2 min if animal fails to maintain a spontaneously beating heart. Squishing sounds from lung during manual compression is indication of lung injury and may be used as a basis for euthanasia.

16. Immediately sample blood gases once animal maintains stable MABP after restoration of spontaneous circulation (ROSC). After 10 minutes of ROSC, decrease respiratory rate from 80 to approx. 60 bpm and %O₂ from 100% to 70% in a mixture with N₂O. Again, sample arterial blood gases.

17. Correct acid-base status with sodium bicarbonate and/or the ventilator settings. (Base excess should be >0; 5-10 is normal). If pH<7.3 inject NaHCO₃ to adjust pH. May need to increase RR to blow off CO₂ if pH is low, in AGS CO₂ is often >60. Stroke volume must be >2mL.

18. Once blood gases are normal, remove arterial and venous catheters by tying off artery and vein with 3 throws of 3-0 silk suture. Close skin with 3-0 Prolene, simple interrupted stitch; at least 3 throws. Inject Sterile Saline (i.p. 1mL/kg). Or, if chronic cannula is needed, go to chronic arterial/venous cannulation SSOP.

19. If IPTT tag is not present, insert an IPTT tag under the skin between the shoulder blades along dorsal midline.

20. Leave on ventilator until spontaneous breathing begins to fight ventilator.

21. Maintain at 37°C (between 36.5 and 37.5°C) for at least 60 min. Animals not able to maintain 36-37.5°C body temperature without heat lamps are placed in a humidified neonatal incubator set at 29°C for 12-24h or until animals regain normal thermoregulation.

22. Place animal in postoperative cage (with underpad and water bottle provided) and put in neonatal incubator set to 29°C once animal spits out endotracheal catheter on their own (or with

some help by the surgeon if catheter is still lodged in throat due to mucus secretions). Do not provide any food in the cage as this can sometimes lead to accidental death.

23. Copy and file anesthesia records, surgery logs, data sheets, animal care log sheets, and neurological deficit scores (NDS) sheets into appropriate binders and locations. Enter blood gas data into Excel, back up LabScribe files to both laptop and designated external hard drive.

24. Monitor a minimum of three times daily per post-op care below until animal shows signs of voluntary feeding, cleaning, and is able to easily move around in cage on their own.

25. Record body temp daily and inspect/clean sutures daily up to seven days and at regular intervals thereafter. Remove sutures at 10-12 days.

Post-Operative Care Procedures

1. Properly sutured incisions do not require post-operative cleaning. However, wounds/sutures are carefully monitored daily for signs of infection, dehiscence, or self-mutilation. Should the latter occur, Veterinary Services will be notified for appropriate follow-up.

2. For 8- 16 h post-op, animals are observed for signs of distress and fed rodent chow soup mixture placed in a petri dish. If not able to eat at all on their own, animals are fed by gavage (1ml/kg body weight) 3 times per day: (The stomach volume is about 3 ml in about 300 g rat and gastric emptying time about 45 - 60 min. Sucrose (i.e., table sugar from grocery store) is mixed ~50:50 with chow and dissolved with tap water to make a dilute solution that can be drawn into a 3 cc syringe using a gavage needle without clogging the needle). Animals that respond and swallow are fed by placing the tip of the gavage needle into the mouth and allowed to consume more than 1mL/kg. Animals not consuming at least 3mL/kg are fed by gavage.

3. Beginning at 8-16 h post-op body temperature is recorded and animals are monitored for neurological impairment and scored per "Neurological Deficit Scores (NDS)" daily for the first seven days and at regular intervals thereafter as specified in research protocol.

4. Vet services will be contacted in case of complications encountered during regular work hours or during after-hour, weekend or holiday care (e.g. wound infection or unusual behavior by animal).

5. Although the severity of brain damage is not expected to produce death, inter-animal variation makes death a possibility. Severity of brain damage may be assessed from neurological examination. Animals showing severe neurological impairment including coma will not be euthanized because eliminating most severely affected individuals will bias final assessment of histology. The number of animals that die will be noted, however, these animals are excluded from histological examination of tissue because death does not allow for in situ fixation of the tissue. Note: Deposit bagged dead animals in refrigerator in AQ office, making sure to put ID number on the bag. Leave a note that the carcass is in the refrigerator, and as always, update record on AQ disposition log and in lab animal inventory notebook.

6. Animals are monitored at least three times per day for signs of voluntary feeding and fed by gavage or "spoon-fed" using a gavage needle that is placed inside their mouth just past their teeth. Contents are delivered into the mouth by gentle pushing while animal swallows voluntarily. In this way animals may be fed ad lib if voluntary swallowing reflexes are present. Animals must consume at least 1mL/kg or will be fed by gavage. Porphyrins are washed from the eyes, nose, and mouth. Sometimes porphyrins are also seen in urine but usually go away within 1-2 days. Animals also may be gently cleaned with wetted gauze if unable to clean on their own. Soiled underpads and/or cotton are changed as needed to avoid soiling the animal and/or incision. Postoperative animals are usually placed on underpads for the first 1-3 days post-surgery and are placed back on shavings when wound is no longer at risk of infection and animal is ambulatory (e.g. hiding under underpad and solely eating dry rodent chow rather than liquefied rat chow soup).

Hemorrhagic Shock SSOP

Preparation of Surgical Site

Shave throat (if tracheotomy is planned), right groin, the area between shoulder blades, and the dorsal surface of both forelegs and a hind leg. Scrub incision sites using 70% alcohol and 100% betadine solution beginning with betadine and alternating with 70% isopropyl alcohol for 3 times (three swabs betadine and two swabs 70% alcohol), ending with 100% betadine. Use circular, inside-to-outside motions when scrubbing incision site so as not to contaminate the innermost region.

Approximately 2.5 cm or less incision is made on the groin region of both the right and left leg to access the femoral vein and artery. The incision is made using skin forceps to lift up the flap of skin and cut with #10 or #15 blade or curved, blunt scissors. Stretching of the incision is made by gently opening/closing the scissors longitudinally along the cut and fascia connective tissue angling downward to lift muscle and expose vessels.

Surgical Procedure

1. Open pressure gauge pack and set up pressure transducer.
2. Prepare injectables and fill three cannulae w/hep-saline with 1cc syringe and 23 ga. leur stub adapter (Becton Dickinson; reorder # 427565) for PE-50; 26ga leur stub for Tygon. Fill one 10cc syringe with sterile saline. Leave sterile ends in pack.
3. Anesthetize with 5% isoflurane and a 30:70 mixture of O₂ (400-500mL/min) and N₂O (1L/min) via scavenger fluovac mask. This insures stable physiological levels for P_{O₂}, P_{CO₂} and pH.
4. Begin endotracheal intubation Open sterile intubation pack. Make sure animal is lying face up on intubation rodent stand and take animal off anesthesia mask and be sure that animal is secured by tooth strap and (optional) sliding bars located on stand itself. Next, insert otoscope with speculum attached and light turned on (otoscope and intubation setup found at

[www.hallowell.com/index.php?pr= Product_Catalogue](http://www.hallowell.com/index.php?pr=Product_Catalogue)) against the palate of the mouth (with the curved slide facing downward) while holding and deflecting the tongue to the side. Next, place a drop of lidocaine gel (Lidocaine Hydrochloride Jelly USP, 2% Rx; 30 mL, NDC 17478-711-30 or equivalent) to the tip of the lidocaine applicator and apply it the epiglottis (arch shaped and located just above the vocal cords) while still holding the otoscope and speculum against the palate of the animal's mouth with the opposite hand. Lidocaine gel application should reduce epiglottis spasm as well as irritation to this sensitive region. Put animal back on anesthesia mask (rats 2.5% isoflurane; AGS 3-3.5% isoflurane) for two minutes.

5. Take animal off mask and reinsert otoscope/speculum unit as described earlier in Step #4. . This time use the endotracheal catheter (14ga 1.75in; BD Insyte Autoguard, shielded iv catheter, ref# 381467) guided with a bent, custom blunt biomedical 17gax4in needle (17TWx3, Popper & Sons, New Hyden Park, NY #7427) or flexible metal stylette attached to a syringe "handle" and try to insert it (with curved side facing up) into the trachea located via the opening between the two vocal cords. Movement of the vocal cords should be slowed down enough in order to maneuver this technique. In rats, the catheter is suitably within the trachea when the orange hub is just outside the incisors; only the orange hub is outside the oral cavity. In AGS, the orange hub should remain within the oral cavity and not extend any farther forward than the back side of the incisors. Immediately check if the catheter is truly in the trachea and not in the esophagus by holding a stainless steel instrument in front of the open end of the catheter (orange hub) so that condensation of respiratory moisture can be seen. If no moisture is visible, then it means that the catheter is in the esophagus. If the first intubation attempt is unsuccessful, repeat the aforementioned steps. Discontinue surgery if more than two tries are needed to execute intubation. Return animal to home cage for at least 1oneweek before repeat attempt.

Suture catheter to lip to avoid dislodging catheter during resuscitation. Close with 3-0 Prolene, simple interrupted stitch with at least 3 throws.

For terminal, non-recovery procedures a tracheotomy may be substituted for oral intubation. The trachea will be exposed as described above. A small hole will be made between the cartilaginous rings in the trachea and the 14ga 1.75in BD Insyte catheter will be inserted between the tracheal rings. The catheter will be secured in the trachea with one or two sutures using 3-0 silk.

6. Shave throat (if tracheotomy is planned), right and left inguinal regions, area between shoulders (if chronic cannulation is planned), and dorsal surface of both forelegs and a hind leg (for ECG electrodes). Scrub animal using 70% alcohol and 100% betadine solution beginning with betadine and alternating with 70% isopropyl alcohol for three times (three swabs betadine and two swabs 70% alcohol), ending with 100% betadine. Use circular, inside-to-outside motions when scrubbing incision so as not to contaminate the innermost region.

7. Ventilate and monitor sO_2 using pulseoximetry. Sample and adjust stroke volume and rate; adjust stroke volume, SV, (typically between 2 – 3 mL) and $\%O_2$ (typically between 450 and 500mL/min) to adjust blood gases (AGS PCO_2 : 30-84mm Hg; P_{O_2} 41-73 mm Hg [P_{CO_2} of 60mm Hg and P_{O_2} of 60mm Hg is typical for AGS]; Rat P_{CO_2} : 35-40; P_{O_2} 100-110 [110-130 is acceptable]. If needed to maintain sO_2 within the normal range for the species, apply 1-5cm PEEP by immersing expiratory tubing into a 5cm high column of water. If arterial pressure begins to fall below a normal value of about 80-120mm Hg, decrease the height of the water column to as low as possible without compromising sO_2 .

8. Record vitals and cover eyes with sterile ophthalmic ointment for lubrication (e.g. vetropolycin, neo-poly-bac, etc.). Locate the temporalis muscle by palpating the small bulge between dorsal midline of the skull and the caudo-dorsal aspect of the eye, and insert the temporalis thermocouple needle 2-4 mm into the muscle with the needle pointing rostrally (toward the nose). Lubricate the rectal thermometer with K-Y jelly and insert 8-10 cm into rectum. Insert EKG leads subcutaneously (ground to hind leg; negative to left front leg, positive to right front leg). Secure

both EKG leads and thermocouples/needles with surgical tape; monitor sO₂ with pulse-oximeter if applicable. Record values on surgery log and anesthesia record.

9. Don sterile gloves, drape and open pack and arrange surgical instruments.

- Small vessel scissors (protect with tip of yellow pipette)
- 45o 5/45 sharp forceps (protect with tip of yellow pipette)
- Blunt; bent 16ga/2.5" needle (may bend, blunt and file needle from IV catheter used for endotracheal catheter) or purchase from Popper&Sons, 17TWx3 custom blunt popper biomedical needle (cat# 7427, <http://popperandsons.com/index.asp>)
- Fine, straight rat-toothed forceps (Miltex 6-106; ~1mm or 1/16" width x 5 inches long)
- 90o curved hemostats (5.5 inch long; Roboz RS 7291)
- 2 x 8 inch smooth inner surface curved (~90o) forceps for teasing apart and holding vessels (Paragon #4004-96 <http://www.paragonmedical.com/>)
- Curved, blunt scissors for cutting skin and separating skin and muscle (Roboz; RS-6891)
- 4 inch curved forceps for holding cannula
- 2 ½" forceps (rough)
- Needle holder
- Sterile drape with holes for throat (if needed) and groin
- (Optional) Y-Leur adapter; used to connect both Epi and NaCHO₃- to venous cannula line for immediate injection during resuscitation

- Group of 6 inch cotton tipped applicators (quantity based on surgeon's preference)
- Small scissors for cutting knots
- ½" pile of gauze wrapped in tin foil (Flush with saline, clean out blood clots, cover with saline wetted gauze)
- (Optional) Extra, 4 x 23ga blunt connectors, 6 x 23ga. Leur stub adapters (for PE-50) 6x 26 ga leur stub adapters (for Tygon tubing)
- 1 silk needle w/suture (for securing endotracheal catheter to lip after intubation)
- 4-7 x 15 inch pieces of silk thread necessary for anchoring and tying off vessels during cannulation
- 2-3 x 3-0 Prolene sutures for skin closure (3/8 19 mm; FS-2, 18" [45 cm] Non-absorbable)
- (Optional) Retractor for throat if doing tracheotomy
- Follow procedure on Excel data sheet

*Note If tracheotomy is planned:

- IV catheter (same for rats and AGS; BD/Insyte; H3124A; 14ga x 1.75 inch; 2.1 x 45mm)
- 3-0 Prolene (when closing)

10. Cannulate the left femoral artery and right femoral vein and artery using polyethylene catheters (Tygon tubing; 0.375-mm ID, 0.75-mm OD; Norton, Akron, OH or PE-50 catheter). Cannula will be introduced ~2cm into each vessel. If a chronic cannulation is required, the

cannula will be inserted approximately 5 to 6 cm into the vessel and externalized at the base of neck (see Chronic Cannulation SSOP, IRBNet #142687-2). The left arterial cannula will be used to record acute blood pressure. Mean arterial blood pressure (MAP) will be measured via the indwelling femoral arterial catheter connected to a precalibrated Statham pressure transducer and will be recorded continuously. The right artery and vein will be used for blood letting and plasma expander infusion, respectively. The rate of hemorrhage will be controlled manually to prevent collapse of the artery due to pressure. The rate and amount of blood withdrawn will be adjusted so that a target MAP is achieved within a set time period (e.g. reaching 35 mm Hg in 15 min of hemorrhage). The volume of blood removed will be recorded. The volume of plasma expander infused will be determined from the volume of blood removed (e.g. replace 50% of the blood removed with plasma expander). The plasma expander will be infused at a constant rate over set period of time (e.g. 5 mL infused over 15 min). Plasma expander will be either Lactated Ringer's solution (LRS) or Ringer's solution. In the event that LRS influences plasma lactate concentration, Ringer's solution will be used as a plasma extender. Blood chemistries (e.g. arterial blood gases, pH, plasma glucose, and plasma lactate) will be measured in microsamples (75-100mL) as needed. Total volume non-hemorrhage blood samples will not exceed 1.5mL.

11. Sample and adjust blood gases (if intubated); adjust stroke volume, SV (typically between 2 – 3 mL) and %O₂ (typically between 450 and 500mL/min) to adjust blood gases (AGS P_{CO2}: 30-84mm Hg; P_{O2} 41-73 mm Hg [P_{CO2} of 60mm Hg and P_{O2} of 60mm Hg is typical for AGS]; Rat P_{CO2}: 35-40; P_{O2} 100-110 [110-130 is acceptable].

12. Monitor T_{rec} and T_{temporalis} until between 36.5 and 37.5°C.

13. Begin blood withdrawal from femoral artery or vein manually. Blood will be withdrawn over 15 min until a target MAP of 35mm Hg is reached. MAP will be maintained at 35-40mm Hg for 20 min by the intermittent withdrawal of blood and/or the infusion of plasma expander.

14. Resuscitate by infusing a set volume of plasma expander (see step 10) over 15 min. The animal will then be monitored for 180 min. 180 minutes following the end of the initial resuscitation animals may undergo a second resuscitation (Resuscitation 2) sufficient to restore the MAP to 80 mm Hg over a period of 15 minutes. The MAP will be held at 80 mm Hg for 10 minutes at the end of resuscitation. Animals in the Sham group will undergo identical anesthetic and surgical procedures but will not be subjected to hemorrhage or resuscitation.

15. Sample blood gases at end of 15 min infusion

16. Correct acid-base status with sodium bicarbonate and/or the ventilator settings. (Base excess should be >0 ; 5-10 is normal). If $\text{pH} < 7.3$, inject NaHCO_3 to adjust pH. Respiratory rate may need to be increased to blow off CO_2 if pH is low. In AGS P_{CO_2} is often >60 . Stroke volume must be $>2\text{mL}$.

17. Once blood gases are normal, remove venous catheters by tying off artery and vein with three throws of 3-0 silk suture. Close subcutaneous tissue with 3-0 Dexon using a continuous stitch. Close skin with 3-0 Prolene, simple interrupted stitch; at least three throws. If tissue collection is to occur after the 180 min monitoring period, catheters will not be removed.

18. Insert IPTT tag, if not present and if animal is to be kept alive for more than 24hrs post hemorrhage.

19. If intubated, leave on ventilator until spontaneous breathing begins and animal starts to fight ventilator.

20. Sample blood gases at the end of Resuscitation 1 and Resuscitation 2.

21. If animal is unable to thermoregulate, place in postoperative cage (with underpad and water bottle provided) and put in neonatal incubator set to 29°C once animal spits out endotracheal catheter on their own (or with some help by the surgeon if catheter is still lodged in throat due to

mucus secretions). Do not provide any food in the cage as this can sometimes lead to accidental death.

22. Copy and file anesthesia records, surgery logs, data sheets, animal care log sheets, and neurological deficit scores (NDS) sheets into appropriate binders and locations. Enter blood gas data into Excel, back up LabScribe files to both laptop and designated external hard drive.

23. Monitor a minimum of three times daily per post-op care below until animal shows signs of voluntary feeding, cleaning, and is able to easily move around in cage on their own.

24. Record body temp daily and inspect/clean sutures daily up to seven days and at regular intervals thereafter. Remove sutures at 10-12 days.

Post-Operative Care Procedures

1. Properly sutured incisions do not require post-operative cleaning. However, wounds/sutures are carefully monitored daily for signs of infection, dehiscence, or self-mutilation. Should the latter occur, Veterinary Services will be notified for appropriate follow-up.

2. For 8- 16 h post-op, animals are observed for signs of distress and fed rodent chow soup mixture placed in a petri dish. If not able to eat at all on their own, animals are fed by gavage (1ml/kg body weight) three times per day. The stomach volume is about 3 ml in about 300 g rat and gastric emptying time about 45 - 60 min. Sucrose (i.e., table sugar from grocery store) is mixed ~50:50 with chow and dissolved with tap water to make a dilute solution that can be drawn into a 3 cc syringe using a gavage needle without clogging the needle). Animals that respond and swallow are fed by placing the tip of the gavage needle into the mouth and allowed to consume more than 1mL/kg. Animals not consuming at least 3mL/kg are fed by gavage.

3. Animals are monitored at least three times per day for signs of voluntary feeding and fed by gavage or "spoon-fed" using a gavage needle that is placed inside their mouth just past their teeth. Contents are delivered into the mouth by gentle pushing while animal swallows voluntarily.

In this way, animals may be fed *ad lib* if voluntary swallowing reflexes are present. Animals must consume at least 1mL/kg or will be fed by gavage. Porphyrins are washed from the eyes, nose, and mouth. Sometimes porphyrins are also seen in urine but usually go away within 1-2 days. Animals also may be gently cleaned with wetted gauze if unable to clean on their own. Soiled underpads and/or cotton are changed as needed.

4. Beginning at 8-16 h post-op body temperature is recorded and animals are monitored for neurological impairment and scored per "Neurological Deficit Scores (NDS)" daily for the first seven days and at regular intervals thereafter as specified in research protocol.

5. Vet Services will be contacted in case of complications encountered during regular work hours or during after-hour, weekend or holiday care (e.g. wound infection or unusual behavior by animal).

6. Severity of MOF makes death a possibility. Animals will be monitored, fed by gavage (1mL/100g body weight) and given supportive care as needed at least 3 times per day. Animals showing severe impairment including coma will not be euthanized because eliminating most severely affected individuals will bias final assessment of histology. The number of animals that die will be noted, however, these animals are excluded from histological examination of tissue because death does not allow for in situ fixation of the tissue. Note: Deposit dead animals in refrigerator in Animal Quarters office, ID# on the bag, leave a note that the carcass is in the refrigerator, and as always, update record on Animal Quarters disposition log and in lab animal inventory notebook.

7. Post-operative animals are usually placed on underpads for the first 1-3 days post-surgery and are placed back on shavings when wound is no longer at risk of infection and animal is ambulatory (e.g. hiding under underpad and solely eating dry rodent chow rather than liquefied rat chow soup).

Chronic Cannulation SSOP

Preparation of Surgical Site

Groin area and area at back of neck will be shaved and washed 3 times with betadine (or generic equivalent). First 2 washes have a 70% isopropyl alcohol rinse. Once the alcohol is dry the final prep is to apply full strength betadine solution by painting it on with a cotton tip swab. This is applied using a circling motion starting at the center of the shaved site and end at the shaved margin

Venous and Arterial Cannulations

For right left leg femoral artery & vein cannulation: surgical scrub & prep of inguinal region from abdominal midline to hock, 3 cm skin incision made (#10 blade, #15 blade, or blunt surgical scissors), blunt dissection of SQ fat until femoral vein, nerve & artery bifurcation located. Blunt dissection of fat towards abdominal wall & along vessel bundle. Proximal artery & vein above bifurcation separated from vessel bundle & cleaned of connective tissue. Using 5-0 silk, distal end of artery is tied off securely with 2 knots. One to two drops of lidocaine (xylocaine) is applied to dilate vessel (if needed). Tiny incision is made into artery using Vannas Spring Scissors-3mm Blades Straight (Fine Sci Tools) & sterile cannula filled with sterile heparinized saline threaded into artery 6 cm. Tiny drop of Nexaband surgical glue placed at cannula entry site into artery to seal. Two 5-0 silk ties made around artery & cannula, each with 5 knots. Distal suture ties then tied around cannula with 5 knots. Patency of cannula checked & blocker put into end of cannula. Procedure repeated for vein cannulation. Two 5-0 Silk sutures made into leg muscle fascia, then tied suture ties around cannulas to secure. Plastic trochar run subcutaneously from surgery site on leg, around to back & up to surgical site between shoulder blades. Assistant made ½ cm skin incision over trochar tip allowing exit of trochar. Loose ends of cannulas threaded up through trochar. Trochar removed, keeping cannulas in place along SQ back & through incision between shoulders. Leg fat placed back over vessel bundle & cannula site. Three 3-0 Dexon simple interrupted sutures placed in fat to hold together. Skin sutured

together with four 3-0 Prolene simple interrupted sutures. Patient rolled over & into ball, cannulas moistened with sterile saline & pushed back into incision between shoulder. Skin incision between shoulders then sutured together with one 5-0 silk suture. "Fingertrap" ties tied around cannulas to secure. Drop of surgical glue to hold knots. Tape butterfly placed on cannulas 1 cm from skin exit. Suture tape butterfly to neck skin with bilateral single 3-0 Prolene sutures. Note: if right femoral artery or vein fail during cannulation, cannulation will be completed on the left femoral artery and/or vein.

Sterile surgical supplies required:

- 2 - pair surgical gloves
- 1 - 6" 2x3 thumb forceps
- 1 - 6" non-toothed forceps
- 1 - 3" angled fine thumb forceps
- 1 - 3" straight fine thumb forceps (fine round tip)
- 2 - 6" curved mosquito hemostats
- 2 - 6" straight mosquito hemostats
- 1 - 6" needle holder
- 1 - 3" tiny ophthalmic scissors to cut vessel with
- 1 - 3" scissors
- 1 - tiny multi-tooth spreader (2 better for obese animals)
- 10 - cotton tipped swabs

- 10 - 2" gauze squares
- 2 - 6" bent glass pipettes with rounded tip
- 1 - plastic trochar with stylette
- 2 - premarked cannulas 45 cm long (35 cm inside animal) Marks at 2, 5, 10, 20, & 30 cm. (Tygon tubing, special order, Saint Gobain PPL Corp)
- 3 - tuberculin syringes
- 2 - #10 scalpel blades
- 2 - #15 scalpel blades
- 1 - 3-0 Dexon/PDS with taper needle
- 1 - 3-0 Surgilene/Prolene with cutting needle
- 6 - 6" pieces of 5-0 silk
- 2 - taper needles for placing silk suture in muscle
- 3 - special 26 ga needles with tips cut (tips filled wit

Wound Closure

Three 3-0 Dexon simple interrupted sutures placed in fat to hold together. Skin sutured together with four 3-0 Prolene simple interrupted sutures. Patient rolled over & into ball, cannulas moistened with sterile saline & pushed back into incision between shoulder blades. Skin incision between shoulders then sutured together with one 5-0 silk suture. "Fingertrap" ties tied around cannulas to secure. Drop of surgical glue to hold knots. Tape butterfly placed on cannulas 1 cm from skin exit. Suture tape butterfly to neck skin with bilateral single 3-0 Prolene sutures.

Post-Operative Care Procedures

Personnel approved for post-operative monitoring will participate in daily post care. Vet services will be contacted in case of complications encountered during regular work hours or during after-hour, weekend or holiday care. After surgery animals are housed in stainless steel cages with small amount of cotton BUT NO wood shavings) and incisions are cleaned once daily with 3% betadine.

Intracardial Perfusion SSOP

Preparation of Surgical Site

Using a #10 scalpel blade or blunt scissors we make about a 6cm ventral midline incision to open the abdomen and thorax. Forceps are used to hold the xiphoid process, a cut is made along the side of the sternum and the sternum is held out of the way with the forceps to allow access to the heart.

Three inches opened along abdominal midline to expose diaphragm and open thoracic cavity.

Surgical Procedure

When blood samples are required blood is sampled from the heart using a 26ga-21ga needle under surgical anesthesia prior to incision.

In some cases, the descending aorta may be clamped to direct flow of fixative to the brain. In other cases when peripheral tissues are to be collected without exposing these tissues to fixative, the animal is bisected just below the diaphragm and one lung is clamped at a bronchi as follows:

An incision is made through the abdominal muscle directly below the rib cage. The incision is expanded laterally until the entire abdominal cavity is opened. The muscles surrounding the spine are cut and the spine and spinal cord are severed. The descending aorta is clamped to prevent blood loss. One lung is clamped off at the bronchi to prevent fixative from perfusing the lung. The upper half of the body is perfused intracardially with saline followed by fixative.

For intracardial perfusion, the right atria is snipped with small scissors (such as iris scissors) to allow efflux of fluid. The apex of the left ventricle is penetrated with an 18 ga needle fit with a sleeve approximately 2-3mm shorter than the needle. The animal is perfused with saline

or heparinized saline (6.7 IU heparin/mL saline) for 1-2 min or longer depending on research objectives (short times for assessing ischemic injury; longer times for preservation of neuronal morphology). After flushing capillaries of blood, perfusion fluid is switched to formaldehyde or paraformaldehyde based fixative for a total of 1-2mL/g body weight depending on experimental protocol. Glutaraldehyde may also be used when fixing for electron microscopy. In other cases, aldehydes may be avoided if they will interfere with tissue analysis. For example, aldehydes are avoided if tissue will be processed for protein carbonyls.

Saline and fixative are pumped at a rate of approximately 80mL/min using a peristaltic pump or by gravity feed.

Time between the start of the abdominal incision to fixation is less than 5 min.

The separation of the lower body from the upper body during bisection allows for swift tissue removal/snap freezing for later RNA/protein analysis. Tissue for RNA analysis should be collected < 5 minutes after death or cessation of blood flow, to insure that RNases do not start to degrade the RNA, etc. In addition, the bisection will insure that the abdominal organs are not perfused with formaldehyde-based fixative which would interfere with RNA and protein analysis.

Appendix C. IACUC Approval

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Institutional Animal Care and Use Committee

808 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

April 15, 2010

To: Kelly Drew
Principal Investigator
From: University of Alaska Fairbanks IACUC
Re: [163051-2] Resistance to multi-organ failure after hemorrhagic shock in arctic ground squirrels

The IACUC reviewed and approved the New Project referenced below by Designated Member Review:

Received:	April 6, 2010
Approval Date:	April 15, 2010
Initial Approval Date:	April 15, 2010
Expiration Date:	April 15, 2011

This action is included on the May 6, 2010 IACUC Agenda.

The PI is responsible for acquiring and maintaining all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol, and could result in revocation of IACUC approval.